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Notice of Opposition to a European Patent

To the 13. Juni 2005
European Patent Office

Tabulation marks

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I. Patent opposed

Opp. No. OPPO (1)

Patent No.	0 656 786
Application No.	93909679.8
Date of mention of the grant in the European Patent Bulletin (Art. 97(4), 99(1) EPC)	15.09.2004

Title of the invention:
USE OF ISOFLAVONE PHYTO-OESTROGEN EXTRACTS OF SOY OR CLOVER

II. Proprietor of the Patent

NOVOGEN RESEARCH PTY LTD.

first named in the patent specification

Opponent's or representative's reference (max. 15 spaces) OPP/AL/13 OREF

III. Opponent

OPPO (2)

Name / Address:
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Multiple opponents: ☐ further opponents see additional sheet

IV. Authorisation

1. Representative
(Name only one representative to whom notification is to be made)

OPPO (9)

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Additional representative(s): ☐ (on additional sheet/see authorisation) OPPO (5)

2. Employee(s) of the opponent authorised for these opposition proceedings under Art. 133(3) EPC

Name(s):

Authorisation(s)

To 1./2.

☐ not considered necessary
☒ has/have been registered under No. 48013
☐ is/are enclosed

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V. Opposition is filed against

— the patent as a whole [X]

— claim(s) No(s).

VI. Grounds for opposition:

Opposition is based on the following grounds:

(a) the subject-matter of the European patent opposed is not patentable (Art. 100(a) EPC) because:

— it is not new (Art. 52(1); 54 EPC) [X]

— it does not involve an inventive step (Art. 52(1); 56 EPC) [X]

— patentability is excluded on other grounds, i. e. Art. [ ]

(b) the patent opposed does not disclose the invention in a manner sufficiently clear and complete for it to be carried out by a person skilled in the art (Art. 100(b) EPC; see Art. 83 EPC). [X]

(c) the subject-matter of the patent opposed extends beyond the content of the application/ of the earlier application as filed (Art. 100(c) EPC, see Art. 123(2) EPC). [ ]

VII. Facts and arguments

(Rule 55(c) EPC)
presented in support of the opposition are submitted herewith on a separate sheet (annex 1) [X]

VIII. Other requests:

IX. Evidence presented

for EPO use only

Enclosed = [X]

will be filed at a later date = [ ]

A. **Publications:**

Publication date

1 WO-A-94/23716 (TUFTS UNIVERSITY SCHOOL OF MEDICINE)

Particular relevance (page, column, line, fig.):
page 1, lines 24-28; page 2, lines 8-10; page 3, lines 1-2, 15-18, 19-33

2 JP-A-59/46217 (RIKEN)

Particular relevance (page, column, line, fig.):
English translation: page 4; table 1

3 JP-A-61/246124 (YAMANOUCHI PHARMACEUTICAL CO. LTD.)

Particular relevance (page, column, line, fig.):
English translation: page 10, lines 20-23; page 2

4 THE LANCET, vol. 339, 1992, page 1233, ADLERCREUTZ H. et al.

Particular relevance (page, column, line, fig.):
whole document

5 J. ENDOCR:, vol. 102 (1), 1984, pages 49-56, AXELSON M. et al.

Particular relevance (page, column, line, fig.):
whole document

6 J. NATL. CANCER INST., vol. 83 (8), 1991, pages 541-6, MESSINA M. et al.

Particular relevance (page, column, line, fig.):
page 542, left column, first three paragraphs; page 545, first paragraph under "Discussic

7 CANCER RESEARCH, vol. 48 (22), 1988, pages 6257-6261, CASSADY J.M. et al.

Particular relevance (page, column, line, fig.):
whole document

Continued on additional sheet [X]

B. **Other evidence**

Continued on additional sheet [ ]

X. Payment of the opposition fee is made

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[x] as indicated in the enclosed voucher for payment of fees and costs (EPO Form 1010)

[ ]

XI. List of documents

Enclosure No.			No. of copies	
0	[x]	Form for notice of opposition	2 (min. 2)	
1	[x]	Facts and arguments (see VII.)	2 (min. 2)	
2		Copies of documents presented as evidence (see IX.)		
2a	[x]	— Publications	2 (min. 2 of each)	
2b	[ ]	— Other documents	(min. 2 of each)	
3	[ ]	Signed authorisation(s) (see IV.)		
4	[x]	Voucher for payment of fees and costs (see X.)	1	
5	[ ]	Cheque		
6	[x]	Additional sheet(s)	2 (min. 2 of each)	
7	[ ]	Other (please specify here):		

XII. Signature of opponent or representative

Place Bresso

Date June 9, 2005

Alessandra Longoni (GA 48013)

Please type name under signature. In the case of legal persons, the position which the person signing holds within the company should also be typed.

CONTINUED

"IX. Evidence presented"

A. **Publications**:

8

FOOD AND COSMETICS TOXICOLOGY, vol. 18 (4), 1980, pages 435-437, DRANE H.M. et al.

Particular relevance (page, column, line, fig.):
whole document

9

J. STEROID BIOCHEM. MOLEC. BIOL., vol. 41 (3), 1992, pages 331-337, ADLERCREUTZ H. et al.

Particular relevance (page, column, line, fig.):
Page 331, "Introduction"

10

AM. J. CLIN. NUTR., vol. 54, 1991, pages 1093-1100, ADLERCREUTZ H. et al.

Particular relevance (page, column, line, fig.):
whole document

11

J. AM. DIETETIC ASS. - PERSPECTIVE IN PRACTICE, vol. 91 (7), 1991, pages 836-840, MESSINA M. et al.

Particular relevance (page, column, line, fig.):
page 837, under "Soybeans and cancer risk", the second paragraph and under "Experimental studies"; page 838 at the beginning of the left column and on the same page at the beginning of the right column.

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WO-A-89/05655 (INNOLAB N.V.)

Particular relevance (page, column, line, fig.):
Whole document

OPPOSITION AGAINST EUROPEAN PATENT No. 0 656 786 B1 IN THE NAME OF NOVOGEN RESEARCH PTY LTD

ZAMBON GROUP S.p.A., a Corporation of Italy, Vicenza, via della Chimica 9, opposes to EP 0 656 786 granted on September 15, 2004, in the name of NOVOGEN Research Pty Ltd on the grounds set forth in Article 100(a) and (b) EPC.

1. GROUNDS OF OPPOSITION

The subject-matter of EP 0 656 786 is not patentable being not new according to Art. 54(1), (2) and (3) EPC and not involving an inventive step according to Art 56 EPC; furthermore, the invention is not disclosed in the patent in a manner sufficiently clear and complete for it to be carried out by a person skilled in the art.

2. THE OPPOSED PATENT

EP 0 656 786 has 11 claims. Claim1 is the only independent claim and remaining claims 2-11 are dependent from claim 1.

Independent claim 1 reads as follows:

> *The use of an isoflavone phyto-oestrogen extract of soy or clover, for the manufacture of a medicament for administration in unit dosage form for the treatment of pre-menstrual syndrome, symptoms associated with menopause, or prostate cancer.*

Claim 2 relates to the use of the above medicament containing a dietary excipient, claims 3-5 more specifically refer to the source of the above extract, claims 6-7 refer to specific isoflavones and to their relative ratios in the above extract, claims 8-11 refer to the dosage, the schedule of treatment, the presence of additional phyto-oestrogens, and the dosage form.

3. THE PRIOR ART

The prior art documents on which the Opponent relies are the following:

D1 - WO-A-94/23716 (TUFTS UNIVERSITY SCHOOL OF MEDICINE)
D2 - JP-A-59/46217 (RIKEN) – with English translation
D3 - JP-A-61/246124 (YAMANOUCHI PHARMACEUTICAL CO. LTD.) – with

English translation

D4 - *THE LANCET*, vol. 339, 1992, page 1233, ADLERCREUTZ H. et al.

D5 - *J. ENDOCR.*, vol. 102 (1), 1984, pages 49-56, AXELSON M. et al.

D6 *J. NATL. CANCER INST.*, vol. 83 (8), 1991, pages 541-6, MESSINA M. et al.

D7 *CANCER RESEARCH*, vol. 48 (22), 1988, pages 6257-6261, CASSADY J.M. et al.

D8 *FOOD AND COSMETICS TOXICOLOGY*, vol. 18 (4), 1980, pages 435-437, DRANE H.M. et al.

D9 *J. STEROID BIOCHEM. MOLEC. BIOL.*, vol. 41 (3), 1992, pages 331-337, ADLERCREUTZ H. et al.

D10 *AM. J. CLIN. NUTR.*, vol. 54, 1991, pages 1093-1100, ADLERCREUTZ H. et al.

D11 *J. AM. DIETETIC ASS. - PERSPECTIVE IN PRACTICE*, vol. 91 (7), 1991, pages 836-840, MESSINA M. et al.

D12 WO-A-89/05655 (INNOLAB N.V.)

4. THE SUBJECT MATTER OF EP 0 656 786 IS NOT NEW

The subject-matter of the opposed patent lacks novelty according to Art. 54(1) and (2) EPC as well as according to Art. 54(3) EPC.

Lack of novelty pursuant to Art. 54(3) EPC

In Opponent's opinion, the opposed patent is not entitled to the claimed priority and therefore D1 is relevant prior art pursuant to Art. 54(3) EPC.

Before discussing the reasons for this lack of novelty, the lack of priority right is discussed herein after.

European patent application No. 93 909 679.8, from which the opposed patent derives, was filed on May 19, 1993 as International patent application PCT/AU93/00230, claiming priority of the Australian provisional application PL2511 filed on May 19, 1992.

International patent application PCT/AU93/00230 (herein after referred to as WO93/23069) does not relate to the same invention of Australian provisional application PL2511 (herein after referred to as AU provisional application) and therefore it is not entitled to the priority date of May 19, 1992.

The AU provisional application has no claims and it is so different in wording from

WO93/23069 that a direct comparison between the two documents cannot be made. Independent claim 1 of WO93/23069 reads as follows:

> *A health supplement comprising a health supplementary amount of a naturally occurring phyto-oestrogen selected from any two or more of genistein, daidzein, biochanin A, formononetin, and/or their glycosides.*

Claims 2-9 depend on claim 1 and independent claim 10 is essentially the same as claim 1 but in a "method of treatment" form.

No doubt that the invention as claimed in WO93/23069 is a phyto-oestrogen containing health supplement characterized by the use of at least two phyto-oestrogens selected from genistein, daidzein, biochanin A, formononetin, and/or their glycosides.
No basis for these specifically selected phyto-oestrogens as essential features of the invention can be found in the AU provisional application.
In fact, it is worth noting that two out of four of the above phyto-oestrogens are never mentioned in the AU provisional application. Reference is made several times to daidzein and genistein but neither biochanin A nor formononetin are ever mentioned.
Referring to the phyto-oestrogens of the invention, the AU provisional application specifically refers to dietary phyto-oestrogens like isoflavones and coumestans, both known for their biological effects (see the paragraph bridging pages 2 and 3 of the AU provisional application).
Through the whole document of the AU provisional application, when a reference is made to the phyto-oestrogens of the invention, the reference is always to isoflavones and/or coumestans and, within the group of isoflavones phyto-oestrogens, reference to daidzein and genistein only is made, daidzein being preferred (see for example the portion of the AU provisional application from page 8, line 14 to page 9, line 21 which cannot be found in WO93/23069).
The phyto-oestrogens of the invention according to WO93/23069 are isoflavones only, some of which never mentioned in the AU provisional application.

Therefore, the invention as described in WO93/23069 refers to the use of some isoflavones as health supplement while the invention as described in the AU provisional application refers to the use of partially different isoflavones and/or

coumestans as health supplement.

In view of the above arguments, the Opponent's opinion is that WO93/23069 and AU provisional application do not refer to the same invention pursuant to Art. 87(1) EPC and no claims of the European patent application from which the opposed patent derives were entitled to the priority of May 19, 1992.

The effective date of the opposed patent for the provisions of Art. 54 EPC is therefore the International filing date of **May 19, 1993** and, consequently, D1 is prior art according to Art. 54(3) EPC.

D1 relates to the use of isoflavonoids from soy beans or other plants for the treatment of menopausal and premenstrual symptoms (see for example page 1, lines 24-28).
On page 2, lines 8-10 and page 3, lines 1-2, the use of a plant extract rich in isoflavonoids is mentioned.
On page 3, lines 28-33, the administration of the isoflavonoids in the form of a dietary product as well as in the form of a medicament in unit dosage form is reported.
In view of the above, claims 1-5 and 11 of the opposed patent lack novelty.
Further, claim 6 of the opposed patent lack novelty in view of D1, page 3, lines 15-18; claims 7-8 lack novelty in view of D1, page 3, lines 19-23; and claim 9 lacks novelty in view of D1, page 3, lines 23-27.

Therefore, claims 1-9 and 11 of the opposed patent lack novelty under Art. 54(3) EPC in view of D1.

Lack of novelty pursuant to Art. 54(1) and (2) EPC

The opposed patent lacks novelty pursuant Art. 54(1) and (2) EPC in view of D2.
D2 relates to carcinostatic agents containing flavonoids or isoflavonoids.
Examples of flavonoids and isoflavonoids specifically include genistein and daidzein (see page 4 and table 1 of D2).
These carcinostatic agents may be administered in the form of an oral or non oral drug.
D2 refers to flavonoids and to isoflavonoids as to known substances so including

their use from natural sources such as plant extracts.
Claims 1, 6 and 11 of the opposed patent lack novelty in view of D2.

The opposed patent lacks novelty pursuant Art. 54(1) and (2) EPC in view of D3.
The carcinostatic activity of genistein together with pharmaceutical compositions containing it in unit or multiple dosage forms is described in D3.
Also clinical dosages of genistein (200 mg to 1000 mg per day) are reported on page 10, lines 20-23 of D3.
The natural origin of genistein as a compound separated from clover is also mentioned on page 2.
Claims 1, 5-6, 8-9 and 11 of the opposed patent lack novelty in view of D3.

5. THE INVENTION OF EP 0 656 786 DOES NOT INVOLVE AN INVENTIVE STEP

The use of isoflavone phytoestrogen extracts from soy or clover for the treatment of pre-menstrual syndrome, menopausal symptoms, or prostate cancer as claimed in the opposed patent lacks inventive step.

The therapeutic use of isoflavone phyto-oestrogens is widely reported in the prior art literature.

As above reported D2 and D3 relate to the use of isoflavonoids as carcinostatic agents.

D4 correlates the effect of dietary phyto-oestrogens on menopausal symptoms in Japanese women. Isoflavonoids such as genistein and daidzein are specifically mentioned and their intake from soy products clearly reported. The Authors conclude that *"High levels of isoflavonoid phyto-oestrogens may partly explain why hot flushes and other menopausal symptoms are so infrequent in Japanese women"*.

D5 relates to soya as a source of equol in man and animals. The study was carried out by using food constituents as well as extracts as a source of phyto-oestrogens (equol precursor). When discussing the results of the study, the Authors suggest to investigate the dietary habits of women with unexplained infertility or disorders of the menstrual cycle and to investigate further whether the presence of phyto-oestrogens in common food-stuffs affects the development and/or treatment of hormone-

dependent tumors.

D6 is a report of a workshop held in June 1990. The role of isoflavones in the prevention of cancer is broadly discussed. In one of the discussed studies (see page 542, left column, first three paragraphs), soy foods and soy extracts were found to be active in inhibiting mammary tumorigenesis. It is interesting to note that one of the tested product, an aqueous alcohol-extracted soy protein concentrate was found inactive because of its low content of isoflavones. As reported in D6 (see page 545, first paragraph under "Discussion"), at the end of the workshop there was a consensus that *"there are sufficient data to justify studying the impact of soybean intake on cancer risk in humans".*

D7 describes the activity of plant extracts, in particular red clover extracts containing isoflavones, as carcinostatic agents.

In D8 soy-bean extracts were tested for oestrogenic activity. All the ethyl acetate extracts (containing genistein and daidzein) were found to be active.

D9 corresponds to the Proceedings of the 10th International Symposium of the Journal of Steroid Biochemistry and Molecular Biology, Recent Advances in Steroid Biochemistry and Molecular Biology, held in Paris on 26-29 May 1991. In the introduction, a brief comment about the literature on lignans (Ligs) and isoflavonoids (Ifls) is reported as follows: *"There is already evidence suggesting that both Ligs and Ifls are protective with regard to BC and that Ifls may be protective with regard to prostate cancer (PC)."* The study relates to the possible mechanisms by which the cancer-protective effect of these compounds is exerted and concludes that the results obtained strongly suggest that lignans and isoflavonoids (from soy products) have cancer-protective properties.

D10 discloses the low mortality in breast and prostate cancer in Japanese women and men, respectively, and this is put into relation with the high intake of soybean products. The role of phyto-oestrogens, in general, and of genistein, in particular, is specifically underlined in connection with the protective activity with respect to prostate cancer.

D11 is an overview of the potential role of soyfoods in cancer prevention. On page

837, under "Soybeans and cancer risk", the second paragraph begins as follows: *"Several compounds with anticancerogenic activity are found in relatively high concentrations in soybeans. Among those thus far identified are isoflavones, protease inhibitors,....."*. Under "Experimental studies", it is specifically mentioned with respect to isoflavones that *"The data of Barnes et al. suggested that the isoflavones in soybeans were responsible for tumor inhibition."* (see page 838 at the beginning of the left column). On the basis of the mechanism of action of isoflavones, on the same page of D11 (see the beginning of the right column), the Authors conclude that *"isoflavones may have a role to play in the prevention of a wide range of cancers"*.

D12 discloses a dietary supplement containing substances, extracted from natural sources, which have a beneficial effect on certain human conditions, particularly menopausal hot flushes. The dietary supplement may be formulated as a beverage or in granule, capsule or suppository form.

In summary, from D2-D11 is known that isoflavones phyto-oestrogens from soy or clover are effective in the treatment of pre-menstrual syndrome, menopausal symptoms, and prostate cancer.

From D5 and D6 is known that soy foods and soy extracts are equally effective, especially in cancer inhibition, provided they are both rich in isoflavones.

D12 shows that it is within the common general knowledge to use a formulation containing an extract from a natural source as valuable alternative to the administration of natural substances through food intake.

From the above, it is clear that the invention of EP 0 656 786 is obvious to a person skilled in the art and therefore does not involve an inventive step.

6. THE INVENTION IS NOT SUFFICIENTLY DISCLOSED IN THE PATENT

The invention as claimed in the opposed patent relates to the use of an isoflavone phyto-oestrogen extract of soy or clover for the preparation of a medicament useful for the treatment of pre-menstrual syndrome, symptoms associated with menopause, or prostate cancer.

Neither the preparation of extracts of soy nor data about their efficacy in the

treatment of the above conditions are reported in the patent.

On the other side, the patent proprietor underlined several times during the examination proceedings that the plant extracts for the use according to the claimed invention are not mere crude extracts and differ also from purified individual isoflavone phyto-oestrogen compounds.

In the absence of any guidance on how to prepare a soy extract useful for the preparation of a medicament as claimed in the opposed patent, the skilled in the art must consider that any soy extract can be used in the claimed invention.

In this case, however, the Opponent wishes to draw to the Opposition Division's attention that the content of isoflavones in soy and clover is different both qualitatively and quantitatively. Also soy extracts and clover extracts are different in the same way.

For example, red clover extracts contains a mixture of four isoflavones, namely genistein, daidzein, biochanin A, formononetin, and in fact also the standardized clover extract contained in Promensil®, the product sold by the patent Proprietor, contains a mixture of genistein, daidzein, biochanin A, formononetin in the biologically active aglycone forms (see *van de Weijer P.H.M. et al., Maturitas 42 187-193* cited as D33 by the patent proprietor during the examining proceedings of the opposed patent).

Soybeans extracts contains only daidzein and genistein.

Biochanin A is known to have estrogenic activity and formononetin has low estrogenic activity but it is converted *in vivo* into equol, an estrogenic metabolite.

The evidences showed by the patent Proprietor to prove the efficacy of a red clover extract in the treatment of pre-menstrual syndrome, symptoms associated with menopause, or prostate cancer cannot prove the efficacy of soy extracts in the same treatment since soy extracts lack of biochanin A and formononetin which are not inert isoflavones components of red clover extracts.

In T 409/91 and T 435/91, the view of the Board of Appeal was that for the requirements of Art. 83 EPC, the disclosure of one way of performing an invention is only sufficient if it allows the invention to be performed in the whole range claimed rather than only in some members of the claimed class.

In Opponent's opinion the claimed invention is not sufficiently disclosed in the opposed patent and, therefore, it does not fulfil the requirement of Art. 83 EPC.

7. CONCLUSIONS

Claims 1-11 of the opposed patent are invalid, since the claimed subject-matter is not novel and/or does not involve an inventive step.
Claims 1-11 are invalid also because the invention is not disclosed in the patent in a manner sufficiently clear and complete for it to be carried out by a person skilled in the art.

The Opponent requests the revocation of the European patent 0 656 786 in its entirety.
In case the Opposition Division does not intend to revoke the opposed patent in its entirety, oral proceedings are requested according to Art. 116 EPC.

D1

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A61K 31/35 — A1

(11) International Publication Number: WO 94/23716

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(30) Priority Data:
08/049,006 16 April 1993 (16.04.93) US

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(74) Agent: CLARK, Paul, T.; Fish & Richardson, 225 Franklin Street, Boston, MA 02110-2804 (US).

(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS

(57) Abstract

A method is provided for preventing or treating symptoms of menopause, premenstrual syndrome, or a condition resulting from reduced levels of endogenous estrogen, by administering to the woman an effective amount of an isoflavonoid. The invention also features a therapeutic dietary product, containing isoflavonoids, for preventing or treating symptoms of conditions resulting from reduced or altered levels of endogenous estrogen.

METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS

Background of the Invention

The present invention relates to therapies for the prevention and treatment of menopausal and premenstrual symptoms.

It has long been recognized that the sharp reduction in endogenous estrogen levels which occurs prior to menopause causes a variety of unpleasant symptoms, e.g., hot flashes, nausea, nervousness, and malaise. Currently, the symptoms of menopause are treated by estrogen replacement therapy, which has recently been shown to increase the risk of certain types of cancer, such as endometrial cancer and breast cancer. Changes in levels of endogenous estrogen may also be responsible for "premenstrual syndrome", a condition occuring in younger women prior to menstruation. Premenstrual symptoms are treated with a variety of hormonal and nonhormonal therapies, which may cause side effects. Safer and more effective therapies for both conditions continue to be sought.

Summary of the Invention

The inventors have found that isoflavonoids, which are constituents of soy beans and other plants, effectively reduce the symptoms of conditions which are caused by reduced or altered levels of endogenous estrogen, e.g., menopause, and premenstrual syndrome. Without being bound by any theory, it is believed that the isoflavonoids bind to estrogen receptors, and thus exert an estrogenic response. These compounds, which are present naturally in soy-based and other plant-based foods, are safe and cause no significant side-effects. Isoflavonoids which may be administered according to the invention include genistein, daidzein, Biochanin A,

formononetin, O-desmethylangolensin, and equol; these may be administered alone or in combination.

Accordingly, in one aspect, the invention features a method of preventing or treating the symptoms of menopause, premenstrual syndrome, or a condition resulting from reduced levels of endogenous estrogen, by administering to the woman an effective amount of at least one isoflavonoid. The isoflavonoid may be administered in any suitable form, e.g., in the form of a plant extract rich in isoflavonoids or in the form of a purified or synthesized isoflavonoid.

In another aspect, the invention features a therapeutic dietary product for preventing or treating symptoms resulting from reduced or altered levels of endogenous estrogen. The dietary product preferably includes a soy extract containing enriched isoflavonoids, provided in a palatable food carrier, e.g., a confectionary bar, biscuit, cereal or beverage.

Other features and advantages of the invention will be apparent from the Description of the Preferred Embodiments thereof, and from the claims.

Description of the Preferred Embodiments

Isoflavonoids are naturally occurring substances, found primarily in soy beans. These compounds are also found in lower concentrations in many other plants. Isoflavonoids can thus be administered to a patient by placing the patient on a diet containing high levels of soy-based food products, e.g., tofu, miso, soybeans, aburage, atuage and koridofu, or other plant products rich in isoflavonoids.

These products may not be readily available in all geographic regions (most of these foods are served predominantly in Japan), and are not be palatable to many women, particularly those accustomed to Western-style food.

Accordingly, an isoflavonoid-containing fraction can be extracted from a soy or plant product. It is preferred that the isoflavonoids be extracted and concentrated from soy bean or soy powder. Isoflavonoids are also available commercially in substantially pure form. The concentrated isoflavonoid is preferably included in a food carrier to form a dietary product. Any type of palatable carrier may be used, but, as the isoflavonoid concentrate has a strong flavor, it is preferred that the carrier include suitable flavorings to impart a different, more palatable flavor. The dietary product may be any type of food product, e.g., a confectionary bar, biscuit, cereal or beverage.

It is preferred that the dietary product contain at least 30 mg/serving total isoflavonoids. The isoflavonoid concentrate included in the dietary product preferably includes a blend primarily comprised of genistein and daidzein. The concentrate typically also contains lower levels of other isoflavonoids. Most preferably, the dietary product contains from about 10 to 30 mg/serving, more preferably about 20 mg/serving of genistein, and from about 5 to 10 mg/serving, more preferably about 7 mg/serving of daidzein. Preferably, a dietary product containing the preferred dosage of isoflavonoids would be consumed at least once per day, preferably 1 to 2 times per day depending upon the severity of the woman's symptoms.

While it is preferred that the isoflavonoid be administered in the form of a dietary product, if desired the isoflavonoid could be administered, preferably in similar dosages, in medicament form, e.g., mixed with a pharmaceutically acceptable carrier to form a tablet, powder or syrup.

Example

The connection between diet and estrogen excretion was studied in Japanese women and men, and in a few children. The women's mean age was 50.4 (SD 18.0) years and they were all from a small village south of Kyoto and consumed a traditional Japanese low-fat diet. Isoflavonoid excretion in the urine was measured in a group of three men, three women, and three children living in Kyoto and consuming the traditional diet. We found a very high excretion of isoflavonoids in the urine of these subjects. The mean values were almost identical in the two groups and especially high excretion was found for genistein (maximum 15.5 umol per 24h in a man) and two other isoflavonoids, daidzein and equol (Table 1). All these compounds bind to estrogen receptors and have weak estrogenic activity. The excretion of the isoflavonoids in urine of the Japanese women was much higher than previously determined levels in American and Finnish women (Table 1). Excretion was high in children as in middle-aged and old people. These compounds were excreted in 100-fold to 1000-fold higher amounts than the levels of endogenous estrogens excreted by normal omnivorous women consuming a western or oriental diet (Table 1).

The excretion of the isoflavonoids in urine was associated with intake of soy products such as tofu, miso, aburage, atuage, koridofu, soybeans, and boiled beans.

It is known that Japanese women have a lower incidence of menopausal symptoms and premenstrual symptoms than the American and Finnish women.

Table 1

Urinary isoflavonoid or estrogen (nmol/day)	Japanese/ Oriental	American	Finnish
Genistein	3440(n=3)	. .	32.1(n=12)
Daidzein	2600(n=10)	216(n=21)	40.5(n=12)
Equol	2600(n=10)	62.8(n=21)	44.2(n=12)
Oestrone (postmenstrual)	4.48(n=9)	. .	4.48(n=10)
Oestradiol (postmenstrual)	0.76(n=9)	. .	0.94(n=10)
Oestriol (postmenstrual)	4.48(n=9)	. .	4.44(n=10)

CLAIMS

1. Use of an isoflavonoid in the preparation of a medicament for preventing or treating a medical condition in a woman caused by reduced or altered levels of endogenous estrogen.

2. The use of claim 1, wherein said isoflavonoid is selected from the group consisting of genistein, daidzein, Biochanin A, formononetin, O-desmethylangolensin and equol.

3. The use of claim 1 wherein said isoflavonoid is in a unit dosage of at least 30 mg.

4. The use of claim 1 wherein genistein and daidzein isoflavonoids are present in said medicament.

5. The use of claim 4 wherein said isoflavonoid comprises from about 10 to 30 mg genistein and from about 5 to 10 mg daidzein.

6. The use of claim 1 wherein said medicament is in the form of a dietary product.

7. The use of claim 6 wherein said dietary product contains at least 30 mg/serving of said isoflavonoid.

8. The use of claim 6 wherein said dietary product is a confectionery bar containing said isoflavonoid.

9. The use of claim 6 wherein said dietary product is a cereal containing said isoflavonoid.

10. The method of claim 6 wherein said dietary product is a biscuit containing said isoflavonoid.

11. The method of claim 6 wherein said dietary product is a beverage containing said isoflavonoid.

12. A dietary product for preventing or treating symptoms of menopause, premenstrual syndrome, or conditions resulting from reduced or altered levels of endogenous estrogen, comprising at least one isoflavonoid provided in a non-soy-based palatable food carrier.

13. The dietary product of claim 12 comprising genistein and daidzein isoflavonoids.

14. The dietary product of claim 12 wherein the food carrier is a confectionery bar.

15. The dietary product of claim 12 wherein the food carrier is a cereal.

16. The dietary product of claim 12 wherein the food carrier is a biscuit.

17. The dietary product of claim 12 wherein the food carrier is a beverage.

18. The dietary product of claim 12 wherein the food carrier contains an amount of the isoflavonoid which is effective in reducing the symptoms.

19. The dietary product of claim 18 comprising at least about 30 mg isoflavonoids per serving.

20. The dietary product of claim 13 wherein said dietary product comprises from about 10 to 30 mg/serving genistein and from about 5 to 10 mg/serving daidzein.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/04189

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) : A61K 31/35-
US CL :514/456, 899
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/456, 899

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS AND CAS ONLINE: ISOFLAVIN?, PMS, ESTRO?, PREMENSTRUAL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US, A, 3,864,362 (FEUER ET AL.) 04 FEBRUARY 1975, COLUMN 1, LINE 33 - COLUMN 2, LINE 44.	1-20 ------ 1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 24 MAY 1994	Date of mailing of the international search report JUL 20 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer KIMBERLY JORDAN Telephone No. (703) 308-1235

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⑲ 日本国特許庁 (JP)　　⑪ 特許出願公開

⑫ 公開特許公報 (A)　　昭59—46217

⑤Int. Cl.³	識別記号	庁内整理番号
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//C 07 D 311/30		7169—4C
311/32		7169—4C
311/36		7169—4C

⑬公開　昭和59年(1984)3月15日

発明の数　1
審査請求　未請求

(全 8 頁)

⑭制癌剤

⑳特　　願　昭57—157103
㉒出　　願　昭57(1982)9月9日
特許法第30条第1項適用　昭和57年3月10日
発行社団法人日本農芸化学会の講演要旨集
(日本農芸化学会昭和57年大会)で発表

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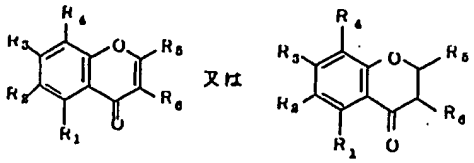
明細書の浄書(内容に変更なし)

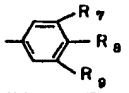
明　　細　　書

1.発明の名称　　制癌剤

2.特許請求の範囲

(1)　一般式



[ただし、式中R_1, R_2, R_3, R_4はH、OH又はOCH_3, R_5, R_6は、  (R_7, R_8, R_9は、H, OH又はOCH_3を示す。)、H, OH又はOCH_3を示す。]

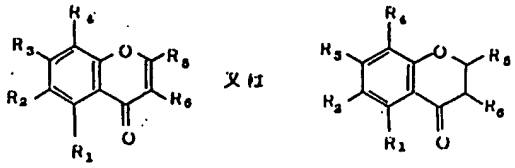
で表わされるフラボノイド又はイソフラボノイド化合物を有効成分として含有することを特徴とする制癌剤。

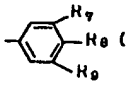
(2)　非経口投与形態による特許請求の範囲第1項記載の制癌剤。

(3)　経口投与形態による特許請求の範囲第1項記載の制癌剤。

3.発明の詳細な説明

本発明は、一般式：



[ただし、式中、R_1, R_2, R_3, R_4は、H, OH又はOCH_3, R_5, R_6は  (R_7, R_8, R_9はH, OH又はOCH_3を示す。)、H, OH又はOCH_3を示す。]

で表わされるフラボノイド又はイソフラボノイド化合物を有効成分として含有することを特徴とする新規な制癌剤に関するものである。

従来、癌化学療法剤として、アルキル化剤(ナイトロジェンマスタード類、エチレンイミン類、スルホン酸エステル類)、代謝拮抗物質(葉酸拮抗

剤、プリン拮抗剤、ピリミジン拮抗剤）、植物性抗分裂剤（コルセミド、ビンブラスチン等）、抗生物質（ザルコマイシン、カルチノフィリン、マイトマイシン等）、ホルモン類（副腎ステロイド、男性ホルモン、女性ホルモン）及びポルフィリン錯塩（マーフィリン、copp）等が用いられている。しかしながら、その殆んどは、細胞毒性の物質であり、重大な副作用を呈するため、低毒性で優れた制癌活性を有する制癌剤の開発が強く望まれている。

そこで、本発明者らは、上記の趣旨に鑑み、低毒性で制癌活性を有する物質について探索、鋭意研究の結果、前記一般式を有するフラボノイド又はイソフラボノイド化合物が動物の腫瘍細胞に対して分化誘導活性を有することを新たに見出し、且つ該物質が著しく低毒性で、優れた制癌活性を有することの新たな知見を得て、本発明の制癌剤を完成するに至つた。本発明の制癌剤の有効成分は、人、家畜、犬、猫等の温血動物に対する優れた癌化学療法剤となり得るものである。

本発明の有効成分であるフラボノイド又はイソフラボノイドとしては、例えば、次の化合物を挙げることができる。

(1) ゲニステイン（Genistein）：4′, 5, 7-trihydroxyisoflavone

(2) ダイゼイン（Daidzein）：4′, 7-dihydroxyisoflavone

(3) フラボン（Flavone）

(4) フイセチン（Fisetin）：3, 3′, 4′, 7-tetrahydroxyflavone

(5) ミリセチン（Myricetin）：3, 3′, 4′, 5, 5′, 7-hexahydroxyflavone

(6) ナリンゲニン（Naringenin）：4′, 5, 7-trihydroxyflavanone

(7) フラバノン（Flavanone）

(8) ノビレチン（Nobiletin）：3′, 4′, 5, 6, 7, 8-hexamethoxyflavone

(9) ケルセチンペンタメチルエーテル（Qvercetinpentamethylether）：3, 3′, 4′, 5, 5′-pentamethoxyflavone

(10) イリゲニン（Irigenin）：3′, 5, 7-trihydroxy-4′, 5′, 6-trimethoxy isoflavone

これらの化合物は、第1表に示す如く、いずれも公知の化合物であり、構造式及び物理的性質は次の通りである（以下、上記化合物番号をもつて示す。）。

表 / 続き

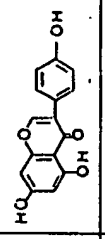
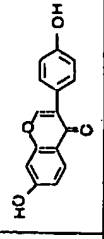
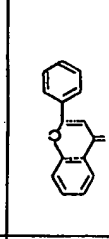
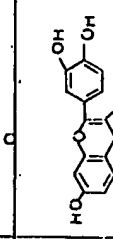
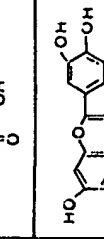
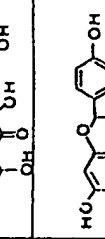
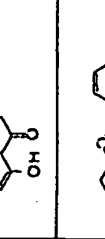
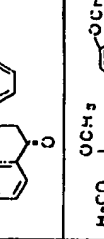
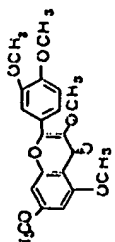
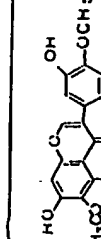
化合物No.	構造式	物理的性質	製造法（文献）
(1)		淡黄色針状晶 m.p.297～298℃（若干分解）	J. Pharm. Chim. (7) 1, 404(1944)
(2)		淡黄色プリズム晶 315～323℃で分解	J. Chem. Soc. 1933, 274
(3)		無色結晶 m.p.99～100℃	Org. Syn. coll. vol. II, 478(1963)
(4)		黄色針状晶 330℃で分解	Ber. 37, 784(1904); J. Chem. Soc. 128, 2334(1926)
(5)		黄色針状晶 m.p.357℃	J. Chem. Soc. 127 181(1925)
(6)		針状晶 m.p. 257℃	ber. 61, 2608(1928); ibid 75, 648(1942)
(7)		無色針状晶 m.p.75～76℃	o-ヒドロキシベンザルアセトフェノンをHCl又はエタノールで処理する。
(8)		淡黄色固体 m.p.130～131℃	Proc. Indian Acad. Sci. 27A 217-22(1948)

表 / 続き

化合物No.	構造式	物理的性質	製造法（文献）
(9)		m.p.150～151℃	Proc. Indian Acad. Sci. 12A 495-497(1940)
(10)		無色板状晶 m.p. 185℃	Tetrahedron Letters n. 5, 61(1960); J. Chem. Soc. (C) 1970, 1219

本発明の制癌剤は、経口及び非経口投与のいずれも使用可能であり、経口投与する場合は、軟・硬カプセル剤又は錠剤、顆粒剤、細粒剤、散剤として投与され、非経口投与する場合は、水溶性懸濁液、油性製剤などの皮下或いは静脈注射剤、点滴剤及び固体状又は懸濁粘稠液状として持続的な粘膜吸収が維持できるように坐薬のような剤型で投与され得る。

本発明の有効成分の製剤化は、界面活性剤、賦形剤、着沢剤、佐剤、及び必要に応じて腸溶性製剤とするために医薬的に許容し得る皮膜形成物質、コーティング助剤等を用いて適宜行うことができ、その具体例を挙げれば、次のとおりである。

本発明の組成物の崩壊、溶出を良好ならしめるためには、界面活性剤、例えばアルコール、エステル類、ポリエチレングリコール誘導体、ソルビタンの脂肪酸エステル類、硬化ヒマシ油、硬化油脂肪アルコール類等の1種又は2種以上を添加することができる。

また、賦形剤として、例えば澱粉、乳糖、デンプン、結晶セルロース、マンニット、軽質無水珪

酸、アルミン酸マグネシウム、メタ珪酸アルミン酸マグネシウム、合成珪酸アルミニウム、炭酸カルシウム、炭酸水素ナトリウム、リン酸水素カルシウム、カルボキシメチルセルロースカルシウム等の1種又は2種以上を組合せて添加することができる。

滑沢剤としては、例えばステアリン酸マグネシウム、タルク、硬化油等を1種又は2種以上添加することができ、また矯味剤及び矯臭剤として、食塩、サッカリン、糖、マンニット、オレンジ油、カンゾウエキス、クエン酸、ブドウ糖、メントール、ユーカリ油、リンゴ酸等の甘味剤、香料、着色料、保存料等を含有させてもよい。

崩壊剤、湿潤剤の如き佐剤としては、例えばココナット油、オリーブ油、ゴマ油、落花生油、乳酸カルシウム、ベニバナ油、大豆リン脂質等を含有させることができる。

また皮膜形成物質としては、セルロース、糖類等の炭水化物誘導体として酢酸フタル酸セルロース（CAP）、またアクリル酸系共重合体、二塩基酸モノエステル類等のポリビニル誘導体としてアクリル酸メチル・メタアクリル酸共重合体、メタアクリル酸メチル・メタアクリル酸共重合体が挙げられる。

また、上記皮膜形成物質をコーティングするに際し、通常使用されるコーティング助剤、例えば可塑剤の他、コーティング操作時の薬剤相互の付着防止のための各種添加剤を添加することによつて皮膜形成剤の性質を改良したり、コーティング操作をより容易ならしめることができる。なお、有効成分を皮膜形成物質を用いてマイクロカプセル化してから賦形剤等と混合した剤型としても良い。

特に代表的な剤型における配合比は下記の通りである。

		特に好ましい範囲
有効成分	0.1～90重量%	0.3～15重量%
賦形剤	10～99.8 〃	85～99.4 〃
滑沢剤	0～50 〃	0～20 〃
界面活性剤	0～50 〃	0～20 〃
皮膜形成物質	0.1～50 〃	0.3～20 〃

特に好ましい賦形剤は、乳糖、結晶セルロース、カルボキシメチルセルロースカルシウムである。

また、投与量は、対象疾患を有効に治療するに十分な量であり、患者の症状、投与経路、剤型などによつて左右されるが、一般に、経口投与の場合、大人では1日当り、約0.01～100mg／kg体重（小人では、0.01～60mg／kg体重）の範囲で、その上限は好ましくは約50mg／kg体重、更に好ましくは約10mg／kg体重程度であり、非経口投与の場合、その上限は約10mg/kg体重程度であり、好ましくは5mg／kg体重、更に好ましくは2mg／kg体重が適当である。

次に、本発明の化合物の細胞活性を抑制した制癌性試験について述べる。

〔1〕フレンド白血病細胞（mouse erythroid leukemia cell，B8細胞）に対する試験

GIBCO製HAMのF-12培地に、15%の牛胎児血清及び60mg／lのカナマイシンを加えたものに、2.5×10^{4}cell／mlとなるようにB8細胞を接種し、これに所定量の被験化合物を加える（最終容量5ml）。

7.5%CO_2中、37℃7日間培養した後、オルキン（Orkin）のベンジジン染色法により染色し、染色された細胞数、すなわち、赤血球への分化によりヘモグロビンを生成するようになつた細胞数を測定し、分化誘導率を求める。

$$\text{分化誘導率 (\%)} = \frac{\text{染色された細胞数}}{\text{全細胞数}} \times 100$$

〔2〕マウス骨髄性白血病細胞（mouse myeloid leukemia cell，M1）に対する試験

GIBCO製イーグルMEM培地に、10%の馬血清及び60mg／lのカナマイシンを加えたものに、5.0×10^{4}cell／ml となるようにM1細

胞を接種し、これに所定量の被験化合物を加える（最終容量5 ml）。

7.5％ CO_2 中、37℃で7日間培養した後、貪食細胞、あるいは顆粒球への分化により誘導されたリゾチーム活性を調べる。なお、リゾチーム活性の1単位（unit）とは、ミクロコッカス・リゾデイクテイカス（Micrococcus lysodeikticus）菌体の懸濁液を基質として、リゾチームを作用させ、pH 6.24、温度25℃で測定し、450 mμ の波長の吸光度を毎分0.001ずつ減少させるようなリゾチームの量をいう。

〔3〕マウス奇形腫細胞（mouse teratocarcinoma）に対する試験：テラトーマ細胞をマウスの腹腔から腹腔へ移植後、1ケ月経過したものを用いた。テラトーマ細胞は、腹腔中では初期胚に似た胚様体（embroid body）という細胞塊として存在し、それらをトリプシン処理などを行うことなく用いた。採取した腹水中で自然沈下させて得られる胚様体をダルベコー変法培地、あるいはハンクス液で3度洗浄後、10％牛胎児血清を含む培地に接種し、所定量の被験化合物を加え、37℃で CO_2 7.5～8％を含む水蒸気を飽和して、空気中で1週間培養する。遠心分離（2000 r.p.m./10分）して得た胚様体を0.86％NaCl溶液で洗浄後、ナフトールAS-MXホスフェートとジアゾ試薬（Fast Violet B Salt）を加えて1時間室温で放置する。これを遠心分離（2000 r.p.m.、10分）して胚様体を分離し、エタノールを加えて1時間室温で放置する。

（未分化の細胞は、赤く着色する）。

これを、535 nm の吸収を測定し、アルカリホスファターゼ活性（分化誘導の程度）を求める。

ヘキサメチレンビスアセトアミド（HMBA）5 mM を加えた場合（アルカリホスファターゼ活性を全く示さない。）を「++」とし、HMBA を加えない場合（アルカリホスファターゼ活性を極めて強く示す。）を「－－」とし、分化誘導の程度を次の段階で示した。

++：アルカリホスファターゼ活性を全く示さない。

+：アルカリホスファターゼ活性をほとんど示さない。

±：アルカリホスファターゼ活性を若干示す。

－：アルカリホスファターゼ活性を強く示す。

－－：アルカリホスファターゼ活性を極めて強く示す。

なお、後述の試験例では、分化誘導作用をもつて、制癌活性を示した。

以下に、本発明を製剤例及び試験例によつて具体的に説明する。

製剤例1（注射・点滴剤）

化合物(1) 10 mg を含有するように粉末ぶどう糖5 g を加えてバイアルに無菌的に分配し、密封した上、窒素、ヘリウム等の不活性ガスを封入して冷暗所に保存する。使用前にエタノール溶解し、0.85％生理的食塩水100 ml を添加して静脈内注射剤とし、1日、10～100 ml を症状に応じて静脈内注射又は点滴で投与する。

製剤例2（注射・点滴剤）

化合物(2) 2 mg を用いて、製剤例1と同様の方法により軽症用静脈内注射剤とし、1日、10～100 ml を症状に応じて静脈内注射又は点滴で投与する。

製剤例3（腸溶性カプセル剤）

化合物(3) 5 g、乳糖2.46 g 及びヒドロキシプロピルセルロース0.04 g を各々とり、よく混合した後、常法に従つて粒状に成形し、これをよく乾燥して篩別してピン、ヒートシール包装などに適した顆粒剤を製造する。次に、酢酸フタル酸セルロース0.5 g 及びヒドロキシプロピルメチルセルロースフタレート0.5 g を溶解して被膜基材となし、前記顆粒を浮遊流動させつゝこの基材を被覆して腸溶性の顆粒剤とする。この組成物をカプセルに充填して腸溶性カプセル製剤100個を製造する。

試験例

第1表の化合物を用い、前記試験法〔1〕，〔2〕及び〔3〕より、フレンド白血病細胞の分化誘導率、マウス骨髄性白血病細胞の分化誘導によるリゾチーム活性及びマウス奇形腫細胞の分化誘導程度を調

べたところ、それぞれ、第2表、第3表及び第4表に示す結果が得られた。

第 2 表

濃度 (μg/ml) ＼ 化合物No.	分化誘導率 (%)										
	(1) *	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	エタノール**	対照
500	35	−	−	−	25	−				<<1	0
250	40	−	−	0	13	−	56	2	28		
125	50	−	−	0	15	−	35	76	25		
63	20	−	15		15	35	64	34	15		
32	8	ca.100	20	<<1	8	20	16		9		
16	14	10	15	8	≦2	8	27	3	6		
8	7	13	10	<3	≦2	1	25		9		
4		<5	<3	<<1	<1	<<1	<10	4	5		

* 分化誘導率同に該当する濃度は、それぞれ400、200、100、50、25、12、6 μg/mlで行つた。

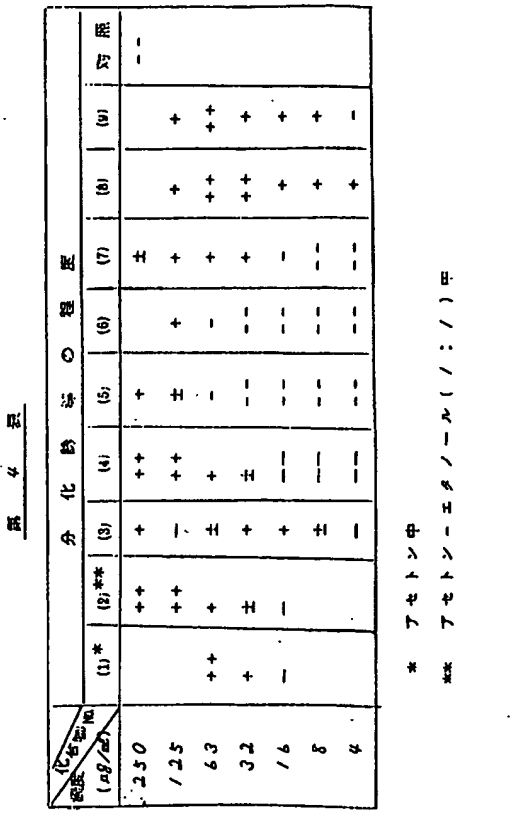
** 比較例20 μl

第 3 表

濃度 (μg/ml) ＼ 化合物	リゾチーム活性 (μg/ml)*					
	(4)	(8)	(9)	(10)	DEX**	対照
500	0				20	132
250	6	10	10			
125	9	10	12	11		
63	25	12	16	11		
32	35	21	15	17		
16	43	19	13	17		
8	12	18	13	17		
4	8	21	13	15		

* 1 μg/ml は、2.2 unit/ml に相当

** 比較例 (positive control) デキサメタゾン 2 μg/ml



上記試験例の結果から明らかなように、本発明の各種フラボノイド及びイソフラボノイドは癌細胞に対して、正常細胞への分化誘導作用を示すとから、毒性の少ない優れた制癌活性を示すことが立証された。

特許出願人　　理化学研究所

手続補正書(方式)

昭和 57.12.
昭和　年　月　日

特許庁長官　殿

1. 事件の表示　昭和 57 年 特許願　第 157103 号

2. 発明の名称　　制癌剤

3. 補正をする者

事件との関係　出願人

名称　(670) 理化学研究所

4. 代理人

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氏名 (5995) 弁理士 中村　　

5. 補正命令の日付　昭和57年11月30日

6. 補正の対象　明細書

7. 補正の内容　別紙の通り

明細書の浄書 (内容に変更なし)

手 続 補 正 書

57.10.-8
昭和 年 月 日

特許庁長官 若 杉 和 夫 殿

1. 事件の表示 昭和 57 年特許願第 15710 号

2. 発明の名称 制 癌 剤

3. 補正をする者

事件との関係 出願人

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氏 名 (5995)弁理士 中 村 稔

5. 補正命令の日付 自 発

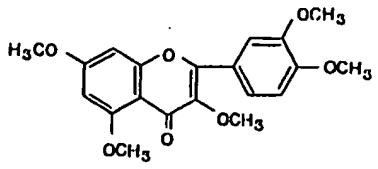
6.

7. 補正の対象 明細書の発明の詳細な説明の欄

8. 補正の内容

明細書第7頁第1表の化合物(9)の構造式を下記のとおり訂正する。

「



」

(19) Japanese Patent Office(JP) (11) Patent Application Laid-Open Publication

(12) Patent Laid-Open Publication (A) S59-46217

(43) Publication Date: March 15, 1984

(51) Int. Cl.[3]	Identification Symbol	
A 61 K 31 / 35	7330 - 4C	
//C 07 D 311 / 30	7169 - 4C	Number of Invention: 1
311 / 32	7169 - 4C	Request for Examination: Not Yet
311 / 36	7169 - 4C	

(Total 8 pgs)

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(54) Title of the Invention CARCINOSTATIC AGENT

(21) Patent Application No. S57-157103

(22) Application Date: September 9, 1982

Article 30, Item 1 of Patent Law is applied; The invention was published in the gist book of Japan Society for Bioscience, Biotechnology, and Agrochemistry on March 10, 1982 (Annual Meeting of Japan Society for Bioscience, Biotechnology, and Agrochemistry in 1982)

(72) Inventor: ISONO, Kiyoshi

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(72) Inventor: ASAHI, Kenichi

1 -4 - 108, Suwahara Danchi, Wako city

(71) Applicant: RIKEN (Rikagaku Knkyusho)

2 ban 1 go, Hirosawa, Wako city

(74) Attorney: Patent Attorney; NAKAMURA, Minoru (other four)

Clean copy of Specification (no change contained)

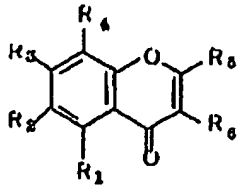
SPECIFICATION

1. Title of the Invention

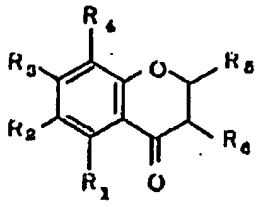
CARCINOSTATIC AGENT

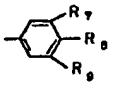
2. Patent Claims

(1) A carcinostatic agent containing a flavonoid compound or an isoflavonoid compound, as an active substance, indicated by the following general formula:



or



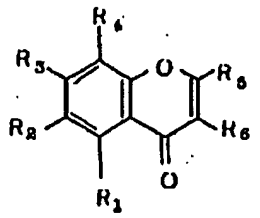
wherein, R_1, R_2, R_3, and R_4 each represents H, OH or OCH_3; R_5, and R_6 each represents  (wherein, R_7, R_8, and R_9 each represents H, OH or OCH_3), H, OH or OCH_3.

(2) A carcinostatic agent as claimed in Claim 1, wherein said carcinostatic agent is administered in the form of a non oral drug.

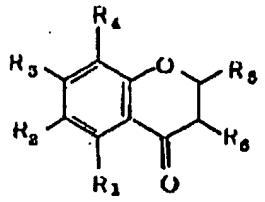
(3) A carcinostatic agent as claimed in Claim 1, wherein said carcinostatic agent is administered in the form of an oral drug.

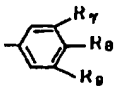
3. Detailed Description of the Invention

The present invention relates to a novel carcinostatic agent containing a flavonoid compound or an isoflavonoid compound, as an active substance, indicated by the following general formula:



or



wherein, R_1, R_2, R_3, and R_4 each represents H, OH or OCH_3; R_5, and R_6 each represents  (wherein, R_7, R_8, and R_9 each represents H, OH or OCH_3), H, OH or OCH_3.

Conventionally, alkylating agents (such as nitrodiene mustards, ethyleneimines, and sulfonic acid esters), metabolic antagonists (such as folic acid antagonist, purine antagonist, and pyrimidine antagonist), plant nuclear division toxicant (such as colcemid, and vinblastine), antibiotic agents (such as sarkomycin, carzinophilin, and mitomycin), hormones (such as adrenocortical steroid, androgenic hormone, and female hormone), and Por phyrin complex acids (such as Marphyrin (manufactured by Dai Ichi Seiyaku), and copp) have been employed as a cancer chemotherapy agent. However, most of those agents are cytotoxic drug type substances and exhibit serious side effects. Therefore, the development of a carcinostatic agent being low toxic and having an

excellent anticancer action has been strongly desired.

Therefore, the present inventors have searched substances being low toxic and having an excellent anticancer action, in view of the purpose in above, eagerly studied and found that the flavonoid or isoflavonoid compound having the general chemical formula previously shown has an induced differentiation activity to tumor cells of animals and exhibits an excellent anticancer action even though being low toxic, thereby having achieved the carcinostatic agent of the present invention. The active substance of the carcinostatic agent according to the present invention may be an excellent cancer chemotherapy agent to warm blooded animals such as human, domestic animals, canines, felines.

Examples of flavonoids or isoflavonoids of the active ingredient according to the present invention may include the following chemical compounds.

(1) Genistein: 4',5,7-trihydroxyisoflavone
(2) Daidzein: 4',7-dihydroxyisoflavone
(3) Flavone
(4) Fisetin: 3,3',4',7-tetrahydroxyflavone
(5) Myricetin: 3,3',4',5,5',7-hexahydroxyflavone
(6) Naringenin: 4',5,7-trihydroxyflavone
(7) Flavanone
(8) Nobiletin: 3',4',5,6,7,8-hexamethoxyflavone
(9) Qvercetinpentamethylether:

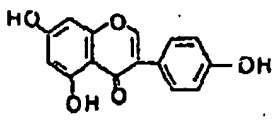
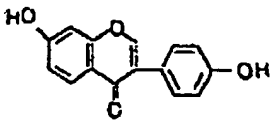
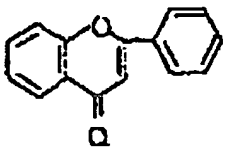
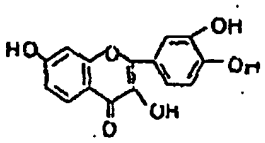
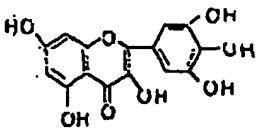
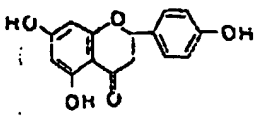
3,3',4,5,5'-pentamethoxyflavone

(10) Irigenin: 3',5,7-trihydroxy- 4',5',6-trimethoxy

Isoflavone

Any of these chemical compounds are well known chemical compounds as shown in Table 1 and their chemical structures and physical properties are as follows (herein after, these chemical compounds are indicated by respective numerical numbers given in above ("chemical compound number").)

Table 1

Chemical Compound No.	Chemical Structure	Physical Property	Method for preparation (Literature)
(1)		Dendritic and needle crystal m. p.: 297 to 298 degrees C (slightly being decomposed)	J. Pharm, Chim. [9] 1,404 (1941)
(2)		Pale yellow colored prism crystal; Being decomposed at a temperature of 315 to 323 degrees C	J. Chem. Soc. 1993, 274
(3)		Colorless crystal m. p.: 99 to 100 degrees C	Org. Syn. Coll. Vol. IV. 478 (1963)
(4)		Yellow colored needle crystal; being decomposed at 330 degrees C	Ber. 37, 784 (1904); J. Chem. Soc. 129, 2334 (1926)
(5)		Yellow colored needle crystal m. p.: 357 degrees C	J. Chem. Soc. 127 181 (1925)
(6)		Needle crystal m. p.: 251 degrees C	Ber. 61, 2608 (1928); ibid 75, 648 (1942)

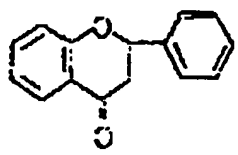
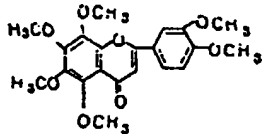
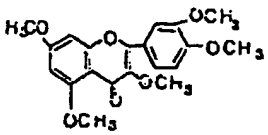
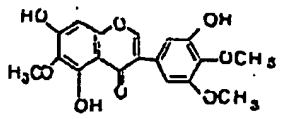
(7)		Colorless needle crystal m. p.: 75 to 76 degrees C	Boiling o-hydroxy benzalacetophenone by HCl or ethanol
(8)		Pale yellow colored crystal m. p.: 130 to 131 degrees C	Proc. Indian Acad. Sci. 27A 217 - 22 (1948)

Table 1 (rest)

Chemical Compound No.	Chemical Structure	Physical Property	Method for preparation (Literature)
(9)		m. p.: 150 to 151 degrees C	Proc. Indian Acad. Sci. 12A 495 - 497 (1940)
(10)		Yellow colored dendritic crystal m. p.: 185 degrees C	Tetrahedron Letters n. 5, 6 (1960); J. Chem. Soc. (C) 1970, 1219

The carcinostatic agent according to the present invention may be administered in the form of any of an oral drug or a non oral drug. When the carcinostatic agent of the present invention is administered in the form of an oral drug, the drug may be administered in the form of soft or hard capsules or tablets, granules, fine grains, powdered drug, while when the present agent is administered in the form of a non oral drug, the drug may be administered

via a hypodermic injection, intravenous injection, intravenous drip in the form of water soluble suspension liquid or oiliness pharmaceutical formulation as well as administered in the form of pharmaceutical form of suppository for the continuous absorption as a solid or suspension viscous liquid through a mucous membrane.

In order to prepare pharmaceutical preparations such as surfactants, diluting agent, lubricant, pharmaceutic adjuvant, and enteric formulation by the active substance according to the present invention, the preparation may be appropriately performed with pharmaceutically acceptable dermatoplasty substances and coating auxiliaries. A specific example thereof is as follows.

To make easier to degrade and elute the composition according to the present invention, one or two or more of surfactants, alcohols, esters, polyethylene glycol derivatives, fatty acid esters of sorbitan and sulfated fatty acid alcohols may be added to the composition.

Moreover, one or two or more of, for example, lactose, starch, crystalline cellulose, mannite, light silicic acid anhydride magnesium aluminate, magnesium aluminate metasilicate, synthetic aluminum silicate, calcium carbonate, sodium hydrogen carbonate, calcium hydrogen phosphate, carboxymethyl cellulose calcium in combination may be added to the composition of the present invention as a diluting agent.

On or two or more of, for example, magnesium stearate, talc, hydrogenated oil may be added as a lubricant to the composition. Moreover, the

composition of the present invention may contain a flavor improvement and a smell improvement such as salt, saccharin, saccharide, orange oil, licorice extra, citric acid, sweetening agent such as glucose, menthol, oil of eucalyptus, and malic acid, flavoring agents, coloring agents, and food preservatives.

Examples of pharmaceutic adjuvant such as suspending agent and wetting agent include, for example, coconut oil, olive oil, sesame oil, peanut oil, calcium lactate, safflower oil, soy bean phospholipids and those may be contained in the composition of the present invention.

Moreover, examples of coating substances include cellulose acetate phthalate (CAP) as a derivative of hydrocarbon such as cellulose and saccharides, methyl acrylate - methacrylate copolymer and methyl methacrylate - methacrylate copolymer as a polyvinyl derivative of copolymers of acrylates, dihydric acid monoesters and the like.

When the above mentioned coating substance is subjected to coating, it is possible to make the properties of the coating agent improved and to make the coating operation easier by adding thereto a conventionally used coating auxiliary, for example, a plasticizer as well as other additives to prevent adhesions between drugs at the coating operation. In addition to the above, the composition of the present invention may be in the form of a pharmaceutical form of a mixture with a diluting agent after the formation of the effective substance into a microcapsule with the use of the coating substance in above.

Especially representative compounding ratio in such a pharmaceutic agent is as follows.

		Especially preferred range
Effective substance	0.1 to 90 % by wt.	0.3 to 15 % by wt.
Diluting agent	10 to 99.8 % by wt.	8.5 to 88.4 % by wt.
Lubricant	0 to 50 % by wt.	0 to 20 % by wt.
Surfactant	0 to 50 % by wt.	0 to 20 % by wt.
Coating substance	0.1 to 50 % by wt.	0.3 to 20 % by wt.

Especially preferred examples of diluting agents are lactose, crystalline cellulose and carboxymethyl cellulose calcium.

The amount of dosage is enough to effectively treat a target tumor and though it depends on symptom of tumor, administrating path, pharmaceutic form and so on, in general, when it is oral administration, approximately 0.01 to 100 mg / kg (body weight) per a day for an adult (for a child, 0.01 to 60 mg / kg (body weight)) with the preferred upper limit is approximately 50 mg / kg (body weight), more preferably approximately 10 mg / kg (body weight). When it is non oral administration, it is appropriate for the dosage that the preferred upper limit is approximately 10 mg / kg (body weight), more preferably 5 mg / kg (body weight), the most preferably 2 mg / kg (body weight).

Next, the tests for verifying the carcinostatic properties of the chemical compound according to the present invention will be described.

[1] Test to Friend leukemia cell (mouse erythroid leukemia cell, B8 cell)

B8 cells were inoculated into HAM F - 12 culture medium, manufactured by GIBCO containing 15 % of a serum of a cattle embryo and 60 mg / l of kanamycin,

so as to obtain 25 $\times$ 10^4 cells / ml. Then, the predetermined amount of each chemical compound to be tested was added thereto (final content of 5 ml).

After the medium was cultured at 37 degrees C for 7 days in 7.5 % of CO_2, the cells were stained by benzidine staining technique of Orkin and the number of stained cells, that is, the number of cells which were differentiated into red cells and became to generate hemoglobin were measured to obtain the rate of induced differentiation.

$$\text{Rate of induced diffentiation} = \frac{\text{Number of stained cells}}{\text{Number of total cells}} \times 100$$

[2] Test to Mouse myeloid leukemia cell (mouse myeloid leukemia cell, M /)

M / cells were inoculated into Eagle MEM culture medium, manufactured by GIBCO containing 10 % of a serum of a horse and 60 mg / l of kanamycin, so as to obtain 5.0×10^4 cells / ml. Then, the predetermined amount of each chemical compound to be tested was added thereto (final content of 5 ml).

After the medium was cultured at 37 degrees C for 7 days in 7.5 % of CO_2, the lysozyme activity induced by the differentiation into macrophage or granulocyte were studied. One unit of lysozyme activity means the amount of the lysozyme with which the absorbency of the wave length of 450 nm is degreased by 0.001 every minute when determined at pH 6.24 and the temperature of 25 degrees C after the activation of the lysozyme with a suspension liquid of Micrococcus lysodeikticus bacterial cells as a matrix.

[3] Test to Mouse tetratocarcinoma (mouse tetratocarcinoma)

The mouse in which tetratomacarcinoma cells were transplanted from a part of peritoneal cavity to another part of peritoneal cavity and left for one month was used. The teratomacarcinoma cells were presented as a cell aggregation of embroid body in the peritoneal cavity which looks like an embryo in the early stage and used them without any treatment such as trypsinization. The embroid body obtained by the natural precipitation in the peritoneal effusion

collected was washed with Dulbecco's Modified Medium or Hanks' solution three times. Then, the cells were inoculated into a culture medium containing 10 % of a serum of a cattle embryo. And the predetermined amount of each chemical compound of the present invention was added to the cells and cultured for one week in the air saturated by water vapor containing 7.5 to 8 % of CO_2 at 37 degrees C. The embroid body obtained by centrifugal separation (2,000 r.p.m. for 10 min.) was washed with 0.86 % of NaCl solution, and naphthol AS - MX phosphate and diazo reagent (Fast Violet B Salt) were added thereto. The mixture was left at a room temperature for one hour. Then, the embroid body is separated from the mixture by centrifugal separation (2,000 r.p.m. for 10 min.) and ethanol is added thereto and left at a room temperature for one hour (undifferentiated cells were colored by red).

The absorbency of the resulting embroid body is determined at the wave length of 535 nm to obtain an activity of alkaline phosphatase (the degree of induced differentiation).

The case of the addition of 5 mM of hexamethylene bis acetamide (HMBA) is indicated by "++" (no exhibition of activity of alkaline phosphatase), the case of the addition of no HMBA is indicated by "--" (extremely strong exhibition of activity of alkaline phosphatase) for the indication of the degree of induced differentiation.

++: no exhibition of activity of alkaline phosphatase

+: almost no exhibition of activity of alkaline phosphatase

+: slight exhibition of activity of alkaline

phosphatase
-: strong exhibition of activity of alkaline phosphatase
--: extremely strong exhibition of activity of alkaline phosphatase

Furthermore, in the following test examples, carcinostatic activities were shown by induced differentiation activities.

The present invention will specifically be explained by examples of pharmaceutical preparations and test ecamples.

Pharmaceutical preparation Example 1 (injection, drop)

Into a vial, 5 g of powdered glucose was dispensed in the germfree condition so as to contain 10 mg of Chemical Compound (1) and sealed and stored in a cool and dark space after inert gas such as nitrogen gas or helium gas was introduced thereinto. Before the use, the pharmaceutical preparation was dissolved into ethanol and 100 ml of 0.85 % saline was added to the dissolved preparation to provide injection. 10 to 100 ml of the injection depending on the symptom is administered per day via an intravenous injection or intravenous drip.

Pharmaceutical preparation Example 2 (injection, drop)

An intravenous injection for mild was prepared with 2 mg of Chemical Compound (2) by the same method of Pharmaceutical preparation Example 1. 10 to 100 ml of the injection depending on the symptom is administered per day via an intravenous injection or intravenous drip.

Pharmaceutical preparation Example 3 (enteric capsule formulation)

Each of 5 g of Chemical Compound (3), 2.46 g of lactose, and 0.04 g of hydroxypropyl cellulose were well mixed and formed into granular in a conventional method. The granular is well dried and sieved to prepare granule suitable for jar package or heat seal package. Next, 0.5 g of cellulose acetate phthalate and 0.5 g of hydroxypropyl methylcellulose phthalate are dissolved to prepare coating base material, and the obtained coating base material is coated to the above granule with floating and flowing to provide enteric granule. Then, the resulting composition is filled into capsules to give 100 enteric capsules.

Test Example

Each Chemical Compound shown in Table 1 was used to study the rate of induced differentiation of Friend leukemia cell, the lysozyme activity by induced differentiation of mouse myeloid leukemia cell, and the degree of induced differentiation of Mouse tetratocarcinoma by the above described test methods [1], [2] and [3] and obtained the results shown in Table 2, Table 3, and Table 4, respectively.

Table 2

Chemical Comp. No. / Conc. (µg / ml)	Rate of induced differentiation (%)										
	(1)*	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	Ethanol**	control
500	35	-	-	-	25	-				<<1	0
250	40	-	-	0	13	-	56	2	28		
125	50	-	-	0	15	-	35	76	25		
63	20	-	15		15	35	64	34	15		
32	8	ca. 100	20	<<1	8	20	16		9		
16	14	10	15	8	≦2	8	27	3	6		
8	7	13	10	<3		1	25		9		
4		<5	<3	<<1	≦2	<<1	<10	4	5		
					<1						

* The tests were performed with concentrations 400, 200, 100, 50, 25, 12, 6 µg / ml, respectively, corresponding to the rate of induced differentiation (%).

** Comparative Example: 20 µl

Table 3

Chemical Comp. No. / Conc. (µg / ml)	Lysozyme activity (µg / ml)*					
	(4)	(8)	(9)	(10)	DEX**	control
500	0				20	132
250	6	10	10			
125	9	10	12	11		
63	25	12	16	11		
32	35	21	15	17		
16	43	19	13	17		
8	12	18	13	17		
4	8	21	13	15		

* 1 µg / ml corresponds to 22 unit / ml

** Comparative Example (positive control) dexamethane 2 µg / ml

Table 4

Chemical Comp. No. / Conc. (µg / ml)	Lysozyme activity (µg / ml)*									
	(1)*	(2)**	(3)	(4)	(5)	(6)	(7)	(8)	(9)	control
250		++	+	++	+		±			--
125		++	-	++	±	+	+	+	+	
63	++	+	±	+	-	-	+	++	++	
32	+	±	+	±	--	--	+	++	+	
16	-	-	+	--	--	--	-	+	+	
8			±	--	--	--	--	+	+	
4			-	--	--	--	--	+	-	

* in acetone ** in acetone - ethanol (1 : 1)

As obvious from the results in the above described tests, every flavonoids and isoflavonoids of according to the present invention exhibits differentiation derivation activities to a normal cell against cancer cell, thereby proving the present invention provides a low toxic and excellent carcinostatic activity.

Patent applicant: RIKEN (Rikagaku Kenkusho)

Amendment (form)

December 5, 1980

To Commissioner of Patent Office

1. Indication of Case: Patent Application No. S57-157103
2. Title of the Invention: Carcinostatic Agent
3. Amended by;
   Patent Applicant
   Name: (679) RIKEN (Rikagaku Kenkusho)

4. Attorney
   Address: 3-1, 3 chome, Marunouchi, Chiyoda ku, Tokyo
   (Tel: 211-8741 (the pilot number))
   Name: (5995) Patent Attorney: NAKAMURA, Minoru (seal)
5. Date of Amendment Order: November 30, 1980
6. Subject of Amendment: Specification
7. Contents of Amendment: as given on the appendix
   Make a clean copy of the specification (no

changes in the contents) (seal)

Amendment (form)

October 8, 1980

To Commissioner of Patent Office, Mr. WAKASUGI, Kazuo

1. Indication of Case: Patent Application No. S57-157103
2. Title of the Invention: Carcinostatic Agent
3. Amended by;

Patent Applicant

Name: (679) RIKEN (Rikagaku Kenkusho)

4. Attorney

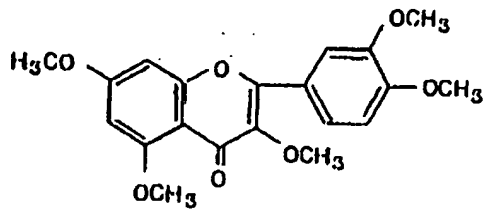
Address: 3-1, 3 chome, Marunouchi, Chiyoda ku, Tokyo

(Tel: 211-8741 (the pilot number))

Name: (5995) Patent Attorney: NAKAMURA, Minoru (seal)

5. Date of Amendment Order: voluntary
6.
7. Subject of Amendment: the column of detailed description of the invention in the specification
8. Contents of the amendment:

Correct the chemical structure of Chemical Compound (9) in Table 7 in page 7 of the specification as follows.


OCH3
H3CO
O
OCH3
OCH3
O
OCH3

[ ]

D3

⑲日本国特許庁(JP)　⑪特許出願公開

⑫公開特許公報(A)　昭61－246124

⑤Int.Cl.⁴	識別記号	庁内整理番号	㊸公開 昭和61年(1986)11月1日
A 61 K 31/35	ADU		
// C 07 D 311/30		6640－4C	

審査請求 未請求 発明の数 1 (全7頁)

㊹発明の名称　制癌剤

㉑特　願 昭60－89770
㉒出　願 昭60(1985)4月24日

㉗発 明 者　小河原　宏　東京都文京区湯島2－33－9
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㉔代 理 人　弁理士 藤野 清也　外1名

明　細　書

1. 発明の名称

制癌剤

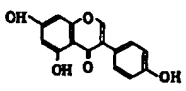
2. 特許請求の範囲

5,7,4'－トリヒドロキシイソフラボン(ゲニステイン)を有効成分とする制癌剤

3. 発明の詳細な説明

(産業上の利用分野)

本発明は 式



で示される 5,7,4'－トリヒドロキシイソフラボン(一般名 ゲニステイン)を有効成分とする制癌剤に関する。

(従来の技術)

ゲニステインは，ジャーナル・オブ・ザ・ケミカル・ソサエティー(Journal of the Chemical Society) 3447頁 1951年に記載されている公知化合物である。同文献によれば，ゲニステインはある種のクローバー(Trifolium subterraneum L.)から単離された化合物で，弱いエストロジェン作用を有することが報告されている。しかし，制癌作用については全く報告されていない。

(発明の作用および効果)

本発明者等は，土壌より分離されたシュードモナス属に属する微生物の発酵生産物中に制癌作用を有する物質を認め，さらに探索した結果，この物質がゲニステインであることをつきとめ発明を完成した。

以下，本発明の化合物の制癌作用および毒性等を説明する。

① 腫瘍細胞増殖阻止作用及びDNA合成阻止作用

ゲニステインの制癌作用を，以下の実験的腫瘍細胞の増殖阻止及びDNA合成阻止試験により調べた。

(イ) ラウス肉腫ウイルスによるラット形質転換細胞(RSV－3Y1細胞)に対する増殖阻止

試験

(ロ) ヒト上皮性癌細胞（A 431 細胞）に対する増殖阻止試験

(ハ) SV 40 ウイルスによるラット形質転換細胞（SV 40 - 3Y1 細胞）に対する増殖阻止試験

(ニ) マウス肥満細胞腫（P815 細胞）に対するDNA合成阻止試験

(ホ) マウス胸腺（EL－4 細胞）に対するDNA合成阻止試験

試験方法および結果

上記(イ)，(ロ)および(ハ)の試験方法は以下の通りである。

(イ) RSV－3Y1 細胞，(ロ) A431 細胞または(ハ) SV 40－3Y1 細胞を2％牛胎児血清（ギブコ（Gibco）社製）及び各種濃度のゲニステインを含むダルベッコー（Dulbecco）のMEM（日本水産㈱製）培地中で培養した。ゲニステインの濃度は無添加，1 μg/ml，3 μg/ml および10 μg/ml の4通りとした。1, 2, 3 および4日後に5％ CO_2 培養器で24時間培養後，[^{3}H] チミジン（Thymidine）（アマシャム・ジャパン㈱製）を0.1 μCi/ウエル添加し，更に18時間培養した。ウエルごとに細胞をグラスファイバーフィルター（ワットマン（Whatman）GF/C）上に取り，フィルターは乾燥後シンチレーションバイアルに入れ，トルエンシンチレーターを加え，液体シンチレーションカウンターで[^{3}H] チミジン（Thymidine）の取り込みを測定した。結果を第2図に示す。

第2図に見られるように，培養液中に，3μg/mlのゲニステインが存在するとP815細胞では約50％チミジンの取り込みが抑えられ，10μg/ml濃度ではP815細胞，EL－4細胞ともにチミジンの取り込みが完全に阻止される。

② チロシン特異的リン酸化酵素活性の阻止作用

ゲニステインの各種酵素活性阻止作用を，以下の3種（a～c）のチロシン特異的プロテインキナーゼ，2種（d，e）のセリン，スレオニンプロテインキナーゼ，及びその他の酵素（f～h）について測定した。

(a) ラウス肉腫ウイルス由来（Src 遺伝子 pp60^{src}）チロシン特異的リン酸化酵素

(b) ヒト上皮性癌細胞増殖因子受容体（EGF レセプター，A431 細胞）チロシン特異的リン酸化酵素

(c) ネコ肉腫ウイルス由来（fes 遺伝子，pp110^{fes}）チロシン特異的リン酸化酵素

(d) c－AMP 依存性プロテインキナーゼ

(e) ホスホリラーゼキナーゼ

(f) ホスホジエステラーゼ

(g) Na^+, K^+－ATPase

(h) 5′－ヌクレオチダーゼ

この中，(a)～(c)は癌遺伝子由来のチロシン特異的リン酸化酵素であり，(d)(e)はセリン，スレオニンのプロテインキナーゼである。

ゲニステインによるこれらの酵素活性阻止作用の測定方法および結果を次に示す。

測定方法

(a) ラウス肉腫ウイルス由来(Src遺伝子 $pp60^{src}$)チロシン特異的リン酸化酵素活性の測定法(エム，エス・コレット，アール，エル・エリクソン：プロシーディング・オブ・ザ・ナショナル・アカデミー・オブ・サイエンシズ・オブ・ザ・ユーエスエー75巻2021～2024頁 1978年参照)

ラウス肉腫ウイルス(RSV)でトランスフォームした3Y1細胞(ラット胎児腎由来線維芽細胞)を培養し，洗浄後それにRIPAバッファー[0.5%NP40，0.1%ソディウムデオキシコレート(sodium deoxycholate)，50mMトリス－塩酸(Tris-HCl) pH 7.2，1mMフェニルメチルスルホニルフルオライド(phenylmethyl sulfonyl fluoride)(PMSF)，0.15M NaCl]を加え，0℃30分間放置することにより可溶化する。これを10万×g 20分間遠心することにより得た上清に，RSVを接種して担癌としたウサギより得た抗血清を加え0℃で30分～1時間インキュベートし，$pp60^{src}$と抗体を反応させる。免疫複合物をプロテインA－セファローズ4B(protein A－Sepharose－4B)(ファルマシア社製)と混合することにより集めてからRIPAバッファーで洗う。得られた$pp60^{src}$－抗体－プロテインA－セファローズ4B複合体を，20mM Pipes－NaOH pH 7.2，5mM $MgCl_2$，1mM DTT，10μM[γ－^{32}P]ATP(2mCi/mmol)中で30℃5分間反応してプロテインキナーゼ反応を行った後SDSを含む反応停止液を加え，3分間煮沸し反応を止める。反応液を8%SDS－ポリアクリルアミドゲルで電気泳動し，オートラジオグラフィののち，切り出した$pp60^{src}$の放射能を液体シンチレーションカウンターにより計測し，リン酸化反応を定量した。

(b) ヒト上皮性癌細胞増殖因子受容体(EGFレセプター，A431細胞)チロシン特異的リン酸化酵素活性の測定法(エス・コウエン，ジイ・カーペンター，エル・キング；ジャーナル・オブ・バイオロジカル・ケミストリー255巻，4834～4842頁1980年参照)

EGFレセプターを多量に含むことの知られているヒト上皮性癌細胞(A431細胞)より調整した細胞膜を酵素源として用いた。50μl中に，20mM Pipes－NaOH pH 7.2，10mM $MgCl_2$，3mM $MnCl_2$，1mM DTT，10μM[γ－^{32}P]ATP(2mCi/mmol)，A431細胞細胞膜(タンパク量10μg)及びゲニステインを含む反応液を5分間反応したのち，反応を停止させ，反応液を8%ポリアクリルアミドゲル電気泳動－オートラジオグラフィで解析して，分子量17万のEGFレセプターのリン酸化の有無を調べる。さらにそのEGFレセプターを切り出し，液体シンチレーションカウンターで放射能を測定することにより，リン酸化の程度を定量した。

・A431細胞からの細胞膜調整法

7%牛胎児血清(ギブコ社製)を含むダルベッコーのMEM(日本水産㈱製)培地で培養したA431細胞を集め，コーエンらの方法(スタンレイ・コーエン，ヒロシ・ウシロ，クリスタ・ストシェック，ミカエル・チンカーズ：ジャーナル オブ バイオロジカル ケミストリー257巻1523－1531頁 1982年参照)により細胞膜小胞を調整した。

(c) ネコ肉腫ウイルス由来(fes遺伝子，$pp110^{fes}$)チロシン特異的リン酸化酵素活性の測定法(アール・エー・フェルドマン，ティー・ハナフサ，エッチ・ハナフサ；セル 22巻757～765頁 1980年参照)

ネコ肉腫ウィルスによりトランスフォームしたラット3Y1細胞，及びこの細胞を接種して担癌としたフィッシャーラットの血清を用いて，$pp60^{src}$の場合と同様にして免疫沈降した$pp110^{fes}$のプロテインキナーゼ活性を測定した。

(d) c－AMP依存性プロテインキナーゼの活性測定法

ウサギ筋肉より調整したc－AMP依存性プロテインキナーゼ（タンパク量4μg）（シグマ（Sigma）社製）を50mM Hepes－NaOH pH 7.5, 10mM $MgCl_2$, 4μM[γ-^{32}P]ATP（2mCi/mmol）, 6mg/mlヒストン type IIA（シグマ社製），10μM c－AMP及びゲニステインを含む反応液50μl中で30℃5分間反応した。2×2cmのワットマンロ紙P81にスポットし，ロ紙を50mM NaClでで5分間ずつ4回洗浄後，さらにアセトンで5分間洗浄し，液体シンチレーションカウンターで放射能を計測した。

(e) ホスホリラーゼキナーゼ活性の測定法

50μl中に40mMトリス－塩酸（Tris－HCl）pH 7.4, 100μM $CaCl_2$, 1mM DTT, 10mM $MgCl_2$, 10μM[γ-^{32}P]ATP（2mCi/mmol），10μgホスホリラーゼb（phosphorylase－b）（シグマ社製），ウサギ筋肉ホスホリラーゼキナーゼ（phosphorylase kinase）（タンパク量2μg）（シグマ社製）及びゲニステインを含む反応液を30℃5分間反応後，SDSを含む反応停止液を加え100℃で2分間煮沸し反応をとめた。ホスホリラーゼbのリン酸化は反応液を8％SDS－ポリアクリルアミドゲル電気泳動－オートラジオグラフィ後，切り出したホスホリラーゼbを液体シンチレーションカウンターで測定することにより定量した。

(f) ホスホジエステラーゼ活性の測定

50μl中に，50mMトリス－塩酸（Tris－HCl）pH 7.5, 8mM $MgCl_2$, 0.8mM EDTA, 0.02mM DTT, 5mM c－AMP（シグマ社製），ウシ心臓ホスホジエステラーゼ（タンパク量10μg）（シグマ社製），及びゲニステインを含む反応液を37℃30分間反応する。

10％TCAを50μl加えて反応をとめ，5,000rpm 10分間遠心して得た上清90μlを用いてリンの定量を行う。リンの呈色反応は，上清液に1％トリトンX－100 5μl，精製水350μl，2.5％モリブデン酸アンモニウムを含む5N－硫酸水溶液50μlを加え20分間放置後，660nmの吸光度を測定することにより定量した。

(g) Na^+, K^+－ATPase活性の測定法

50μl中に，50mMトリス－塩酸（Tris－HCl）pH 7.5, 60mM NaCl, 25mM KCl, 2mM $MgCl_2$, 0.1mM EDTA, 3mM ATP, イヌ腎臓より調整したNa^+, K^+－ATPase（タンパク量560ng）及びゲニステインを含む反応液を37℃30分間反応後，ホスホジエステラーゼと同様にして反応の結果生じたリンを定量した。

・Na^+, K^+－ATPaseの調製

Na^+, K^+－ATPaseは，カワムラらの方法（カワムラ，オータ，ナガノ：ジャーナルオブバイオケミストリー87巻1327－1333頁1980年参照）イヌ腎臓外髄（outer medulla）を50mMイミダゾールpH 7.4, 0.25Mスクロース，1mM EDTA, 0.1mM ATPを含むバッファー中でポリトロン（polytron）（キネマティカ（Kinematica）社製）で破壊後超遠心することにより得られたミクロソーム画分をSDSで抽出することにより調製した。

(h) 5′－ヌクレオチダーゼ活性の測定法

50μl中に55mMトリス－塩酸（Tris－HCl）pH 8.5, 5.5mM $MgCl_2$, 1.1mM ATP, 10mM 酒石酸ナトリウムカリウム塩，5′－ヌクレオチダーゼ（蛇毒）（シグマ社製）及びゲニステインを含む反応液を，37℃3分間反応後，ホスホジエステラーゼと同様にして反応生物のリン酸を定量した。

結　果

ゲニステインの各酵素に対する活性阻止作用

酵素系	ID_{50} (μg/ml)
(a) pp60^{src}プロテインキナーゼ	0.8
(b) EGFレセプタープロテインキナーゼ	0.7
(c) pp110^{fps}プロテインキナーゼ	6.5

(d) c-AMP依存性プロテインキナーゼ	>100
(e) ホスホリラーゼ キナーゼ	>100
(f) ホスホジエステラーゼ	>100
(g) Na^+, K^+ - ATPase	>100
(h) 5'-ヌクレオチダーゼ	>100

ID_{50}：50%阻止量

以上の結果に示されるように，グニステインは癌遺伝子由来のチロシン特異的リン酸化酵素活性を特異的に阻止する。

チロシン特異的リン酸化酵素は，癌細胞の増殖に関与すると考えられているから，この酵素活性の特異的阻止作用が認められたことは，グニステインの制癌作用を裏付けるものである。

③ C57BL/6 系マウスを用い，グニステインを腹腔内に注射して急性毒性を調べた。LD_{50} は 500mg/kg 以上であった。

上記 腫瘍細胞増殖阻止作用，DNA 阻止作用およびチロシン特異的リン酸化酵素活性の阻止作用の試験結果より，グニステインはすぐれた制癌作用を有しており，しかも急性毒性の結果も低いので，ヒトおよび動物の癌の治療，癌の転移に伴う疾患の治療および再発の予防のための制癌剤として有用である。

グニステインの臨床投与量は活性成分として，通常成人1日当り，200～1.000mgであり，これを1～4回に分けて投与する。投与量は患者の状態や年令等，個々の場合に応じて適宜調節される。

グニステインは単独で治療に供されるほか，他の化学療法剤あるいは免疫療法剤と併用される。併用される化学療法剤としては，サイクロホスファミド，ビンブラスチン，ビンクリスチン，アドリアマイシン，6-メルカプトプリン，5-フルオロウラシル，マイトマイシンC，ブレオマイシン，アクラシノマイシン，ネオカルチノスタチン，シトシンアラビノシド，シスプラチン，アクチノマイシンD，ニトロソウレア系薬剤等が挙げられる。

また，免疫療法剤としては，たとえば，クレスチン，BCG，ピシバニール，レンチナン，インターフェロン，インターロイキン等が挙げられる。これらの薬剤と併用する場合の投与量はグニステイン1に対し，併用薬剤 0.001～10 程度が適当である。

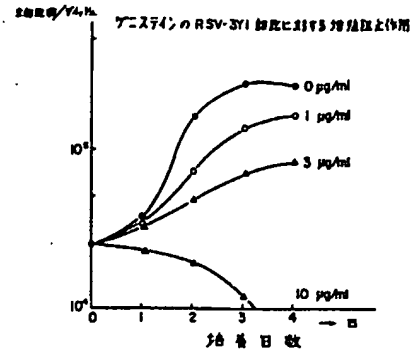
グニステインの投与は，経口剤（錠剤，カプセル剤，液剤）あるいは非経口剤（直腸投与製剤，注射剤，ペレット）の製剤形態で行なわれる。これ等の製剤は，任意慣用の製剤用担体あるいは賦形剤を通常の方法によって配合された組成物として調製される。この際使用される担体あるいは賦形剤は，一般的に用いられるもので良く，たとえば，錠剤の場合，水，ブドウ糖，乳糖，アラビアゴム，ゼラチン，マンニトール，でん粉ペースト，マグネシウムトリシリケート，タルク，トウモロコシでん粉，グラチン，コロイドシリカ，馬鈴薯でん粉，尿素等が利用できる。また液剤は，水性または油性の懸濁液，溶液，シロップ，エリキシル剤であってもよく，これらは通常の方法で調製される。直腸投与のためには，坐剤用組成物として提供され，基剤としては，通常用いられるもの，たとえばポリエチレングリコール，ラノリン，カカオ脂，ウイテプゾル® （ダイナミットノーベル社）等を使用できる。

4. 図面の簡単な説明

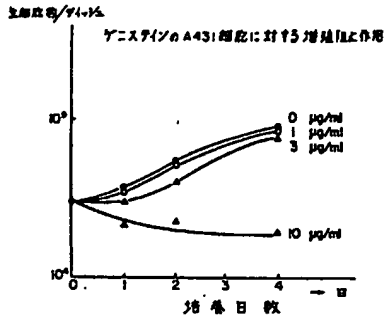
(1) 第1図(イ)，(ロ)および(ハ)はグニステインのRSV-3Y1細胞，A431細胞およびSV40-3Y1細胞に対する増殖阻止作用を示す。

(2) 第2図はグニステインのP815およびEL-4細胞に対するDNA合成阻止作用を示す。

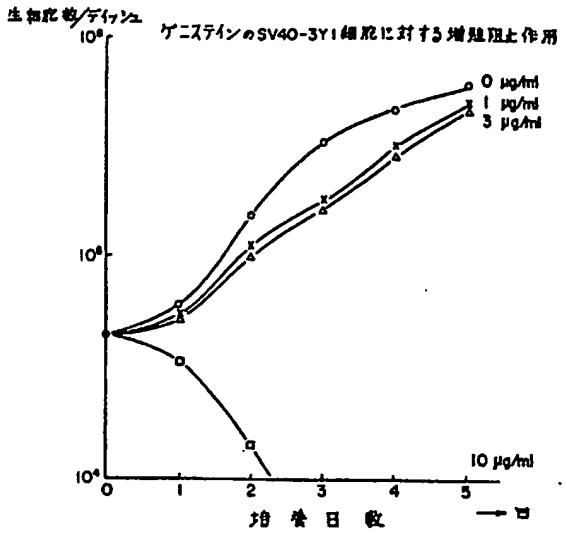
第 1 図 (イ)



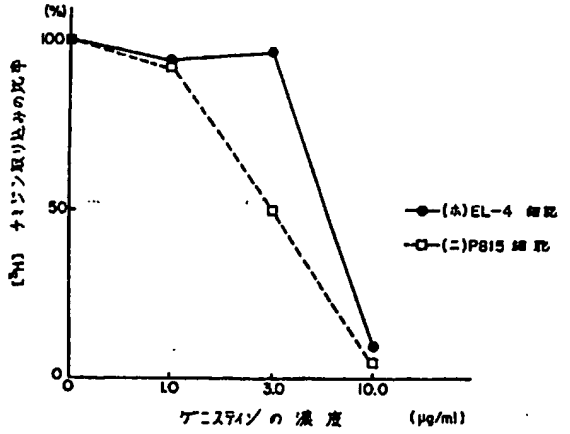
第 1 図 (ロ)



第 1 図 (ハ)



第 2 図



手 続 補 正 書 (自発)

昭和60年5月23日

特許庁長官 志 賀 学 殿

1. 事件の表示
昭和60年特許願第89770号

2. 発明の名称
制癌剤

3. 補正をする者
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5. 補正の対象
明細書の「発明の詳細な説明」の個

6. 補正の内容
別紙の通り

特許庁 60.5.25 出願第二係

方式審査 (杉本)

(1) 明細書第4頁第2行「(イ)～(ロ)」を「(イ)～(ヘ)」に訂正する。
(2) 明細書第6頁第3行「Ｓｒｃ」とあるを「ｓｒｃ」に訂正する。
(3) 明細書第7頁2行、「Ｓｒｃ」とあるを、「ｓｒｃ」に訂正する。
(4) 明細書第8頁第10行「反応して」を「反応させて」に訂正する。
(5) 明細書第9頁7行「四整」とあるを「四製」に訂正する。
(6) 同頁下から第1行、「四整」とあるを、「四製」に訂正する。
(7) 明細書第10頁8行、「四整」とあるを「四製」に訂正する。
(8) 明細書第11頁3行、「四整」とあるを「四製」に訂正する。
(9) 同頁第10行「反応した。」を「反応させた。」に「ロ紙」を「濾紙」に訂正する。
(10) 同頁第11行「ロ紙」を「濾紙」に訂正する。
(11) 同頁第11行「で」を削除する。
(12) 明細書第13頁10行「四整」を「四製」に訂正する。
(13) 明細書14頁11行「3分間」を「30分間」に訂正する。

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SPECIFICATIONS

1. Title of the Invention

Carcinostatic Agent

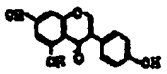
2. Claims

A carcinostatic agent whose active principle is 5,7,4'-trihydroxyisoflavone (genistein)

3. Detailed Description of the Invention

Applicable field of industry

This invention relates to a carcinostatic agent whose active principle is 5,7,4'-trihydroxyisoflavone (commonly known as genistein), and which is represented by the formula



Prior art

Genistein is a known compound, recorded in 1951 on page 3447 of the *Journal of the Chemical Society*. According to this article, genistein is a compound separated from a type of clover (*Trifolium subterraneum L.*), and is reported to have a weak oestrogen effect. However, absolutely no carcinostatic action is reported.

Action and effects of the present invention

The inventors of the present invention recognized a substance in the fermentation products of microorganisms belonging to the *Pseudomonas* genus isolated from soil as having

carcinostatic action and, as a result of further research into the substance, determined that the substance was genistein, and perfected the present invention.

The following describes the carcinostatic properties and toxicity of the compound of the present invention.

← Tumour cell propagation inhibiting action and DNA synthesis inhibiting action

The carcinostatic properties of genistein were investigated through the following experiments on the inhibition of the propagation and inhibition of the synthesis of DNA in the following experimental tumour cells.

(a) Tests on inhibiting propagation of rat cells transformed with Rous' sarcoma virus (RSV-3Y1)
(b) Tests on inhibiting propagation of human epidermal carcinoma cells (A431 cells)
(c) Tests on inhibiting propagation of rat cells transformed with SV40 virus (SV 40-3Y1 cells)
(d) Tests on inhibiting synthesis of DNA in mouse mast cell carcinomas (P815 cells)
(e) Tests on inhibiting synthesis of DNA in mouse thymuses (EL-4 cells)

Test methods and results of tests

The tests (a), (b) and (c) above were performed in the following manner.

The RSV-3Y1 cells (a), A341 cells (b) and SV40-3Y1 cells (c) were cultured in *Dulbecco MEM* (manufactured by Nippon Suisan KK) containing 2% calf embryo serum (manufactured by Gibco) and varying concentrations of genistein. Four levels of genistein concentration were employed: none added, 1 μg/ml, 3 μg/ml and 10 μg/ml. The numbers of live cells per dish were counted by means of Trypan Blue after the first, second, third and fourth days. The results are set out in Figure 1 (a) to (b).

As can be seen from Figure 1, genistein was found to have the effect of arresting cell propagation at levels of addition of from 1 μg/ml to 3 μg/ml, and the effect of inhibiting cell propagation was very strong at a level of addition of 10 μg/ml.

The methods of tests (d) and (e) above were as follows.

P815 cells (d) and EL-4 cells (e) were suspended in RPMI 1640 culture (manufactured by Nippon Suisan KK) to which 2% 56° C 30 minute inactivated calf embryo serum (manufactured by Flow Laboratories) and 80 μg/ml gentamycin (Manufactured by Essex (Japan) KK) had been added, and a final cell count of 2×10^5 cells/ml was produced. 200 μl per well of the cell suspension solution was placed in a 96 well flat-based microplate (manufactured by Sumitomo Bakelite), and genistein was added in the following concentrations: none, 1 μg/ml, 3 mg/ml and 10 μg/ml. The plate was cultured for 24 hours in a 37° C 5% CO_2 culture system, whereupon 0.1 μCi/well of [^{3}H] Thymidine (manufactured by Amersham (Japan) KK) was added, and the solutions were cultured for a further 18 hours. The cells in each well were collected by glass fibre filter (Whatman GF/C) , and the filters were dried and placed in scintillation vials, toluene scintillator was added, and the uptake by [^{3}H] Thymidine was measured by means of liquid scintillation counter. The results are set out in Figure 2.

As can be seen from Figure 2, when 3 μg/ml of genistein was present in the culture solution, the uptake of Thymidine by the P815 cells was restricted to approximately 50%, while at a level of 10 μg/ml, the uptake of Thymidine by the P815 and also EL-4 cells was completely inhibited.

↑ Inhibiting action against tyrosine-specific phosphorylase

The inhibiting action of genistein against various enzymes was measured in relation to the following three types of tyrosine-specific protein kinase (a to c), two types of serine and threonine protein kinase (d and e) and other enzymes (f to h).

(a) Tyrosine-specific phosphorylase derived from mouse carcinoma virus (Src gene $pp60^{src}$)

(b) Human epidermal carcinoma cell propagation factor receptor (EGF receptor, A431 cell) tyrosine-specific phosphorylase

(c) Tyrosine-specific phosphorylase derived from cat carcinoma virus (fes gene, $pp110^{fes}$)

(d) c-AMP dependent protein kinase

(e) Phosphorylase kinase

(f) Phosphodiesterase

(g) Na^+, K^+-ATPase

(h) 5'-nucleotidase

Of these, (a) to (c) related to tyrosine-specific phosphorylase derived from carcinoma genes, and (d) and (e) to serine and threonine protein kinase.

The method of measurement of the inhibiting action of genistein on the enzyme activity of these, and the results of such measurements, are shown below.

Method of measurement

(a) Method of measurement of activity of tyrosine-specific phosphorylase derived from Rous' sarcoma virus (Src gene $pp60^{src}$) (see M.S. Collet and R.L. Elligson: *Proceedings of the National Academy of Sciences of the USA*, vol.75 pp2021-2024 (1978))

3Y1 cells (fibroblasts derived from rat embryo kidney) transformed by Rous' sarcoma virus (RSV) are grown, and after washing with RIPA buffer (0.5% NP40, 0.1% sodium deoxycholate, 50 mM Tris-HCl pH 7.2, 1 mM phenylmethyl sulphonyl fluoride (PMSF), 0.15M NaCl) is added, and the cells are solubilized by being allowed to stand

for 30 minutes at 0° C. This is centrifuged for 20 minutes at 100,000 x g and is then inoculated with RSV, antiserum obtained from carcinoma-infected rabbits is added and the mixture is incubated for from 30 minutes to 1 hour at 0° C and the $pp60^{src}$ and antibodies are reacted together. The immune complex is concentrated by mixing with protein A-Sepharose-4B (manufactured by Pharmacia) and is then washed in RIPA buffer. The $pp60^{src}$-antibody- protein A-Sepharose-4B complex so formed reacts for 5 minutes at 30° C in 20 mM Pipes-NaOH pH 7.2, 5 mM $MgCl_2$, 1 mM DTT and 10 μm [γ-^{32}P] ATP (2 mCi/mmol), the protein kinase reaction is performed, whereupon a reaction halting solution containing SDS is added, the mixture is boiled for 3 minutes and the reaction is halted. The reaction solution is subjected to electrophoresis with 8% SDS-polyacrylamide gel, and after autoradiography, the radiation from the $pp60^{src}$ is measured by means of a liquid scintillation counter, and the phosphorylation reaction is quantified.

(b) Method of measurement of activity of tyrosine-specific phosphorylase from human epidermal carcinoma cell proliferation factor receptor (EGF receptor, A431 cell) (See S. Komin G. Carpenter, and L. King: *Journal of Biological Chemistry*, vol. 255, pp.4834-3842 (1980))

Cell membranes prepared from human epidermal carcinoma cells (A431 cells) that are known to contain large numbers of EGF receptors are used as the enzyme source. A reaction solution containing genistein, 20 mM Pipes-NaOH pH 7.2, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 1 mM DTT, 10μM (γ-^{32}P) ATP (2mCi/mmol) and A431 cell membrane (protein content 10 μg) is allowed to react together in 50 μl for 5 minutes, whereupon the reaction is halted, whereupon the reaction solution is subjected to electrophoresis with 8% SDS-polyacrylamide gel, and after analysis by autoradiography, the EGF receptors of molecular weight of 170,000 were examined for the presence of phosphorylation. The EGF receptors were further isolated, and the radiation was measured by liquid scintillation counter, and the extent of phosphorylation was measured.

- Method of preparation of cell membranes from A431 cells

A431 cells propagated in *Dulbecco* MEM (manufactured by Nippon Suisan KK) containing 7% calf embryo serum (manufactured by Gibco) were collected, and cell membrane follicles were prepared by the method of Rowen et al. (See Stanley Rowen, Hiroshi Ushiro, Krista Stosiek and Michael Cingaz: *Journal of Biological Chemistry*, vol. 257, pp.1523-1531 (1982)).

(c) Method of measurement of activity of tyrosine-specific phosphorylase from cat sarcoma virus (fes gene, $pp110^{fes}$) (See R.A. Feldman, T. Hanafusa and H. Hanafusa: *Cell*, vol. 22, pp.757-765 (1980))

Rat 3Y1 cells transformed with cat sarcoma virus and these cells were inoculated, and serum from cancer-bearing Fisher rats was used, and the protein kinase of immune precipitated $pp110^{fes}$ was measured after the same manner as for $pp60^{src}$.

(d) Method of measurement of activity of c-AMP-dependent protein kinase

c-AMP-dependent protein kinase prepared from rabbit muscle (protein content 4 μg) (manufactured by Sigma) reacts for 5 minutes at 30° C in 50 μl of a reaction solution containing 50 mM Hepes-NaOH pH 7.5, 10 mM $MgCl_2$, 4 μM [γ-^{32}P] ATP (2 mCi/mmol), 6 mg/ml histone type IIA (manufactured by Sigma), 10 μm c-AMP and genistein. This was spotted onto 2 x 2 cm Whatman filter paper P81, the filter paper was rinsed four times for 5 minutes on each occasion in 50 mM NaCl, and then was rinsed again with acetone, and the radiation was measured by means of a liquid scintillation counter.

(e) Method of measurement of phosphorylase-kinase activity

40 mM tris-HCl pH 7.4, 100 μm $CaCl_2$, 1 mM DTT, 10 mM $MgCl_2$, 10 μM [γ-^{32}P] ATP (2 mCi/mmol), 10 μg phosphorylase-b (manufactured by Sigma), rabbit muscle phosphorylase kinase (protein content 2 μg) (manufactured by Sigma) and genistein were reacted together in a 50 μl reaction solution for 5 minutes at 30° C, whereupon a reaction halt solution containing SDS was added, and the solution was boiled for 2 minutes at 100° C to halt the reaction. The phosphorylation of the phosphorylase-b was measured by 8% SDS-polyacrylamide gel electrophoresis - autoradiography of the reaction solution, followed by measurement of the separated phosphorylase-b by means of a liquid scintillation counter.

(f) Measurement of the activity of phosphodiesterase

50 mM tris-HCl pH 7.5, 8 mM $MgCl_2$, 0.8 mM EDTA, 0.02 mM DTT, 5 mM c-AMP (manufactured by Sigma), cow heart phosphodiesterase (protein content 10 μg) (manufactured by Sigma) and genistein in 50 μl in a reaction solution were reacted for 30 minutes at 37° C.

50 µl of 10% TCA was added and the reaction was halted, the solution was centrifuged for 10 minutes at 5,000 rpm and 90 µl of the supernatant so derived was measured for phosphorus. The phosphorus colour reaction was measured by 660 nm absorbance after the addition to the supernatant solution of 3 µl of 1% *Triton X-100*, 350 µl of distilled water and 50 µl of 5N aqueous sulphuric acid containing 2.5% ammonium molybdate and allowing to stand for 20 minutes.

(g) Measurement of the activity of Na^+, K^+ Tpase

Na^+, K^+ Tpase was prepared by the method of Kawamura et al. (see Kawamura, Ota and Nagano: *Journal of Biochemistry*, vol. 87, pp.1327-1333 (1980)): The outer medulla of dog kidney was ground in a buffer containing 50 mM imidazole pH 7.4, 0.25 M sucrose, 1 mM EDTA, and 0.1 mM ATP by polytron (manufactured by Kinematica) and then was ultracentrifuged to provide a microsome fraction that was extracted by means of SDS.

(h) Measurement of the activity of 5'-nucleotidase

A reaction solution containing 55 mM tris-HCl pH 8.5, 5.5 mM $MgCl_2$, 1.1 mM ATP, 10 mM potassium sodium tartrate, 5'-nucleotidase (snake venom) (manufactured by Sigma) and genistein was reacted in 50 µl for 3 minutes at 37° C, and the phosphorus levels of the reaction product were measured in the same manner as for phosphodiesterase.

Results

Inhibiting action of genistein in relation to various enzymes

Enzyme	ID_{50} (µg/ml)
(a) $pp60^{src}$ protein kinase	0.8
(b) EGF receptor protein kinase	0.7
(c) $pp110^{fps}$ protein kinase	6.5
(d) c-AMP dependent protein	>100

kinase	
(e) Phosphorylase kinase	>100
(f) Phosphodiesterase	>100
(g) Na^+, K^+-ATPase	>100
(h) 5'-nucleotidase	>100

ID_{50}: Level at which 50% inhibition occurs

As is clear from the above results, genistein has a specifically inhibiting action against tyrosine-specific phosphorylase derived from carcinoma genes.

Tyrosine-specific phosphorylase is believed to contribute to the propagation of carcinoma cells, and hence the recognition of a specific inhibiting effect against the action of this enzyme is the background to the carcinostatic effect of genistein.

→ C57BL/6 strain mice were injected in the abdominal cavity with genistein, and the acute toxicity of the genistein was examined. LD_{50} was found to be not less than 500 mg/kg.

In view of the above results of tests on its inhibiting effect on the propagation of tumour cells, its inhibiting effect on DNA, and its inhibiting effect on tyrosine-specific phosphorylase, genistein has an excellent carcinostatic effect, and moreover possesses a low toxicity, and thus is of value as a carcinostatic in the treatment of carcinomas in humans and animals, in the treatment of symptoms associated with metastasis of carcinomas, and in the prevention of relapse in carcinoma cases.

The clinical dosage of genistein is from 200 mg to 1000 mg of the active component per adult per day, administered in from one to four doses. The amount administered may be adjusted appropriately according to the individual circumstances of the patient, such as condition and age and so forth.

Genistein may be administered in isolation or in combination with other chemical treatment agents or immunological agents. Chemical treatment agents that may be employed in combination with genistein include cyclophosphamide, vinblastine, vincristine, adriamycin, 6-mercaptopurine, 5-fluorouracil, mytomycin C, pleomycin,

aclasinomycin, neocarzinostatin, cytosine arabinomide, actinomycin D, and nitrosourea and so forth. Immunological agents that may be employed in conjunction with genistein include for example creatine, BCG, [illegible], lentinan, interferon and interleukin and so forth. When genistein is employed in conjunction with other pharmaceuticals, the dosage of genistein is appropriately 1 of genistein to between 0.001 and 10 times the pharmaceutical employed in conjunction.

The dose of genistein may be prepared in form for oral administration (tablet, capsule or liquid) or non-oral administration (for rectal administration, inoculation or pellet). Such dose of genistein may be prepared as a combination with any commonly employed carrier or vehicle blended in the normal manner. Any generally employed carrier or vehicle may be employed, such as for example, in the case of tablets, water, fructose, lactose, gum arabic, gelatine, mannitol, starch paste, magnesium trisilicate, milk, maize starch, colloidal silica, potato starch or urea and the like. In liquid form, an aqueous or oleaginous suspension, solution, syrup, or elixir may be employed, and thee are prepared in the normal manner. For rectal administration, the genistein may be supplied as a suppository, and the base may be any normally employed base such as for example, polyethylene glycol, lanolin, cocoa fat, or *Witefzol*® (manufactured by Dynamit-Nobel) and so forth.

4. Simplified description of the drawings

(1) Figure 1 (a), (b) and (c) show the inhibiting effects of genistein on the propagation of RSV-3Y1 cells, A431 cells and SV40-3Y1 cells.

(2) Figure 2 shows the inhibiting effects of genistein on DNA synthesis in P 815 cells and EL-4 cells.

Figure 1 (a)

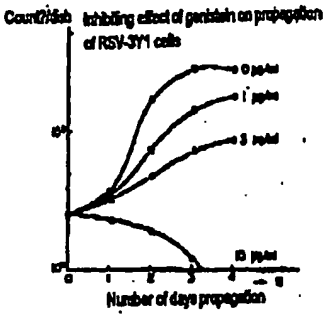


Figure 1 (b)

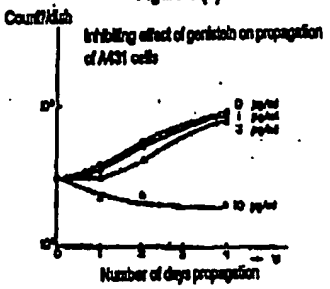
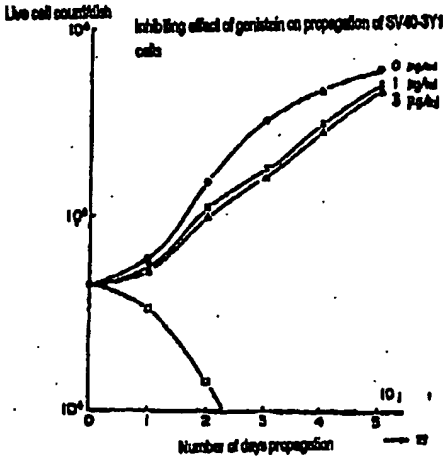


Figure 1 (c)



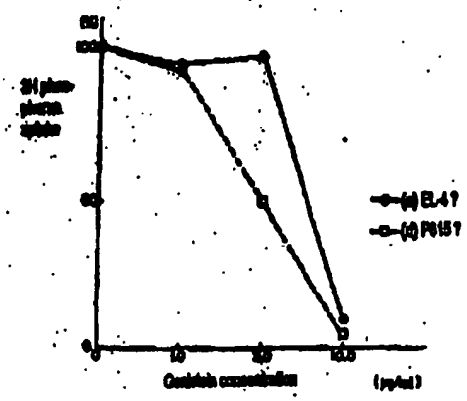


Figure 2

Amendment (Voluntary)

May 23 1985

Mr Manabu Shiga
Director, Patent Agency

1. Statement of Matter
Patent Application No. 89770 of 1985

2. Title of Invention
Carcinostatic Agent

3. Person making Amendment
Standing in matter: Patent Applicant
Name: Yamanouchi Pharmaceutical Co. Ltd
Address: 1-5 banchi, Nihonbashimachi 2-chome, Chuo-ku, Tokyo 103
Representative Shigeo Morioka (and another)

4. Agent
Name: Kiyoya Fujino (and another)
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5. Subject of Amendment
Detailed Description of Invention within Specifications

6. Contents of Amendment
See attached

(1) [English] Page 2 line 35 '(a) to (b)' changed to '(a) to (c)'

(2) [English] Page 3 line 19 'Src' changed to 'src'

(3) [English] Page 3 line 39 'Src' changed to 'src'

(4) [English] Page 4 line 4-5 'reacts' changed to 'is reacted'

(5) [Not relevant in English translation]

(6) [Not relevant in English translation]

(7) [Not relevant in English translation]

(8) [Not relevant in English translation]

(9) [English] Page 5 line 4 'reacts' changed to 'is reacted'; [Not relevant in English translation]

(10) [Not relevant in English translation]

(11) [Not relevant in English translation]

(12) [Not relevant in English translation]

(13) [English] Page 6 line 5 '3 minutes' changed to '30 minutes'

in newborn babies support an essential role for n-3 fatty acids in retinal development.[5]

The DHA content of erythrocytes is small compared with that of grey-matter, but the fatty acid composition of the erythrocyte membranes may indicate the fatty acid status of neural and perhaps other membranes. During the period of most rapid DHA accumulation in the developing rat, diet-induced changes in neural DHA are reflected in red blood cell DHA.[6]

Dietary n-3 fatty acids can also modify endogenous prostaglandin production and perhaps by this means influence uterine prostaglandins and gestation time. In the Faroe Islands, where birthweights are amongst the highest in the world with long gestation periods and rapid fetal growth, the intake of marine fat rich in n-3 fatty acids is high and erythrocyte DHA values in pregnant women were found to be almost twice those in normal individuals in other countries.[7]

The lower erythrocyte DHA found in patients on epoetin could be due to an increased requirement for this n-3 fatty acid as a result of increased red cell production, and this implies a deficiency of or a rate-limited production of DHA. However, plasma DHA values were not low, which raises the possibility of a defect of incorporation of this fatty acid into the membrane in patients on haemodialysis.

A low membrane DHA probably has little effect on red cell function and may be of minor importance in adults, although it is of interest that visual hallucinations have been described in patients on epoetin.[8] Unlike the adult, the fetus requires DHA in quantity for its developing nervous system, and haemodialysed patients do occasionally become pregnant. For reasons not fully understood, pregnancy in uraemia is associated with a high risk of premature labour and retarded fetal growth.[9] A lack of DHA would be detrimental to the fetus, and our results indicate that in a uraemic pregnant woman on haemodialysis, low quantities of membrane DHA could be one of the hazards to which the fetus is exposed. Because epoetin gives rise to even lower membrane DHA content, its use could increase the risk to the fetus: n-3 fatty acid dietary supplements are indicated.

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Dietary phyto-oestrogens and the menopause in Japan

SIR,—Lock, in an article on the menopause,[1] has discussed differences between Japanese women and women in western societies. Japanese women have a much lower frequency of hot flushes than women in Canada. Lock concluded that "cultural indifference to the hot flush in Japan" was unlikely to account fully for these findings.

Recently our Helsinki group studied, in collaboration with Japanese scientists, the diet and phyto-oestrogen excretion in Japanese women and men, and in a few children.[2] The women's mean age was 50·4 (SD 18·0) years and they were all from a small village south of Kyoto and consumed a traditional Japanese low-fat diet. We studied a group of three men, three women, and three children living in Kyoto and consuming the traditional diet, and in this group we measured the isoflavonoid genistein.[2] We found a very high excretion of phyto-oestrogens in urine. The mean values were almost identical in the two groups and especially high excretion was found for genistein (maximum 15·5 μmol per 24 h in a man) and two other isoflavonoids, daidzein and equol (table). All these compounds bind to oestrogen receptors and have weak oestrogenic activity.[3] The excretion of the isoflavonoids in urine of the Japanese women was much higher than in American and Finnish women (table) (ref 4 and unpublished data) and as high in children as in middle-aged and old people. These compounds were excreted in 100-fold to 1000-fold higher amounts than those of endogenous oestrogens in normal omnivorous women consuming a western or oriental diet (table).

URINARY EXCRETION OF ISOFLAVONOID PHYTO-OESTROGENS AND ENDOGENOUS OESTROGENS IN JAPANESE OR ORIENTAL WOMEN, AND IN AMERICAN AND FINNISH OMNIVOROUS WOMEN

Urinary isoflavonoid or oestrogen	Japanese/ Oriental	American	Finnish
Genistein	3440 (n = 3)*	..	32·1 (n = 12)
Daidzein	2600 (n = 10)*	216 (n = 21)	40·5 (n = 12)
Equol	2600 (n = 10)*	62·8 (n = 21)	44·2 (n = 12)
Oestrone (postmenopausal)	4·48 (n = 9)†	..	4·48 (n = 10)
Oestradiol (postmenopausal)	0·76 (n = 9)†	..	0·94 (n = 10)
Oestriol (postmenopausal)	4·48 (n = 9)†	..	4·44 (n = 10)

All assays by gas chromatography/mass spectrometry in selected ion-monitoring mode with deuterated internal standards.[5,6] Women collected two to four 72 h urine samples 3–6 months apart and values are thus means of urinary excretion in individual subjects over 6–12 days. Results as geometric means in nmol/24 h.
*Values from ref 2.
†Oriental postmenopausal women (recent immigants to Hawaii). Same women as in ref 7, but oestrogens measured by new technique.[6]

The excretion of the isoflavonoids in urine was associated with intake of soy products such as *tofu, miso, aburage, atuage, koridofu,* soybeans, and boiled beans. All isoflavonoids are weak oestrogens and such high amounts could have biological effects, especially in postmenopausal women with low oestrogen levels. High levels of isoflavonoid phyto-oestrogens may partly explain why hot flushes and other menopausal symptoms are so infrequent in Japanese women.

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Soya – a dietary source of the non-steroidal oestrogen equol in man and animals

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ABSTRACT

The dietary origin of the weak oestrogen equol (7-hydroxy-3-(4'-hydroxyphenyl)-chroman) present in human urine has been investigated using gas chromatography–mass spectrometry. Feeding experiments with different food constituents and monitoring the urinary excretion of equol revealed that soya food yields more than 0·1 mg urinary equol/g flour ingested. From this source the glucoside of daidzein (4',7-dihydroxyisoflavone) has been isolated and identified as a precursor of equol. Both equol and daidzein were characterized as monoglucuronide conjugates in human urine and the concentration of urinary equol exceeded the concentrations of the classical oestrogens by 100- to 1000-fold after ingestion of a single meal containing soya protein. The potential biological significance of this result is discussed.
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INTRODUCTION

Equol (7-hydroxy-3-(4'-(hydroxyphenyl)-chroman) is an isoflavan possessing weak oestrogenic activity, in the order of 10^{-3} times that of oestradiol (Braden, Hart & Lamberton, 1967; Shutt & Braden, 1968), while also being an antioestrogen in binding competitively with oestradiol to uterine cytosol receptors (Shutt & Cox, 1972; Tang & Adams, 1980). The compound was first discovered in the urine of pregnant mares over half a century ago (Marrian & Haslewood, 1932) and then in goat (Klyne & Wright, 1957), cow (Klyne & Wright, 1959), hen (MacRae, Dale & Common, 1960; Common & Aimsworth, 1961), sheep (Braden *et al.* 1967; Shutt & Braden, 1968) and rat (Axelson & Setchell, 1981). In these animals equol is formed by intestinal bacterial degradation of phyto-oestrogens present in the feed (Batterham, Hart, Lamberton & Braden, 1965; Nilsson, Hill & Davies, 1967; Shutt & Braden, 1968; Batterham, Shutt, Hart *et al.* 1971; Axelson & Setchell, 1981). Ingestion of larger quantities of clover, particularly *Trifolium subterraneum*, which has a high content of equol precursors, leads to an infertility syndrome in sheep referred to as clover disease, in which a cystic condition in the reproductive tract is accompanied by a failure to conceive (Bennetts, Underwood & Shier, 1946; Moule, Braden & Lamond, 1963; Morley, Axelsen & Bennett, 1964; Shutt, 1976).

The occurrence of equol in the urine of man was only recently reported (Axelson, Kirk, Farrant *et al.* 1982). The amounts excreted were similar to the endogenous oestrogens, but were not related to any hormonal status.

The dietary origin of equol in man is not known, but here we report that soya protein has a remarkably high content of an equol precursor which has been isolated and identified as the glucoside of the isoflavone, daidzein (4',7-dihydroxyisoflavone). This phyto-oestrogen is converted into equol, conjugated with glucuronic acid and is then excreted in urine.

MATERIALS AND METHODS

Urine samples

Urine (24 h) collections were obtained from a healthy man (age 34 years) and woman (age 25 years) and from 20 mature female rats (~200 g) of the Sprague–Dawley strain. The urine was collected in

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polyethylene flasks, frozen immediately and stored at −20 °C until analysed.

Diets

Rats were fed commercial pelleted food (Astra-Ewos, Södertälje, Sweden) or a semisynthetic diet, D7 (Midtvedt & Gustafsson, 1981), composed of wheat starch, casein, arachis oil, salts and vitamins. Food constituents (2–5 g/24 h) and equivalent amounts of extracts were tested for the presence of equol precursors by adding them to the semisynthetic diet in exchange for starch or oil and feeding it to one to five rats for 2 days. Soya flour (Soyolk; Soya Foods Ltd) was obtained from A/B Risenta, Stockholm, Sweden (composition: 40% protein, 20% fat and 20% carbohydrate). The two human subjects were given lunch meals in which 40 g of commercial textured soya (Natural Protoveg; Direct Foods Ltd, Petersfield, Hants; composition: 52% protein, 1% fat and 31·5% carbohydrate) was substituted for meat over a 5-day period. The soya was cooked according to the manufacturer's instructions.

Isolation of daidzein from soya

Soya flour (5 g) was refluxed for 1 h with 125 ml 80% aqueous ethanol. After filtration and evaporation of the alcohol, non-polar lipids were removed by first washing the aqueous extract with hexane (30 ml) and then passing it through a column bed (4 × 0·8 cm) of Lipidex 1000 (Packard Instrument Co., Downers Grove, Illinois, U.S.A.) (Dyfverman & Sjövall, 1978) in water. The aqueous effluent (about 30 ml) was then extracted with a Sep-Pak C_{18} cartridge (Waters Associates Inc., Milford, Maryland, U.S.A.) which was washed with 10 ml water before elution with 8 ml methanol (Shackleton & Whitney, 1980). The eluate was passed through a column bed (4 × 0·4 cm) of the strong cation exchanger sulphohydroxypropyl Sephadex LH-20 (SP-LH-20, H^+) (Axelson & Sjövall, 1979) and the material in the methanolic effluent (13 ml) was separated into neutral and phenolic fractions on a column bed (4 × 0·4 cm) of the strong anion exchanger triethylaminohydroxypropyl Sephadex LH-20 (TEAP-LH-20, OH^-) (Axelson, Sahlberg & Sjövall, 1981). Phenolic compounds, eluted from the column with 8 ml CO_2-saturated methanol, were further fractionated by preparative thin-layer chromatography (TLC) on a Merck precoated plate (silica gel 60 F_{254}, 20 cm × 20 cm, Merck, Darmstadt, West Germany) with ethylene chloride : acetic acid : water (10 : 10 : 1, by vol.). Spots were located by inspection under a u.v. lamp at 254 nm or for analytical TLC by spraying with a solution of H_2SO_4 : ethanol (1 : 1, v/v) and heating at 80–100 °C. Major components were scraped from the plate, eluted and rechromatographed on TEAP-LH-20 for removal of TLC impurities. After hydrolysis with β-glucosidase (Emulsin; Sigma Chemical Co., St Louis, Missouri, U.S.A.; 25 units in 5 ml 0·1 M-acetate buffer, pH 5, at 37 °C for 24 h), deconjugated compounds were extracted using a Sep-Pak C_{18} cartridge as described above.

Extraction and isolation of equol from urine

Equol was extracted from urine (1–6 ml) with a Sep-Pak C_{18} cartridge (Shackleton & Whitney, 1980) and conjugates, eluted with 8 ml methanol, were hydrolysed with *Helix pomatia* juice (Reactifes IBF Soc. Chim., Pointet Girard, Villeneuve la Garenne, France; 30 000 Fishman units β-glucuronidase in 5 ml 0·2 M-acetate buffer, pH 4·5) at 62 °C for 1 h (Scholler, Métay, Herbin & Jayle, 1966). After extraction on a Sep-Pak C_{18} cartridge, liberated equol in 8 ml methanol was purified by passage through a column bed (4 × 0·4 cm) of SP-LH-20 (H^+) (Axelson & Sjövall, 1979) and chromatography on a column (4 × 0·4 cm) of TEAP-LH-20 (OH^-) (Axelson *et al.* 1981). A phenolic fraction was obtained by elution of the latter column with 8 ml methanol saturated with CO_2. After removal of the CO_2 by application of vacuum, water was added to give a final concentration of 72% methanol and the sample passed through a column bed (2 × 0·4 cm) of diethylaminoethyl (DEAE)-Sephadex (Pharmacia, Uppsala, Sweden) in base form (Axelson *et al.* 1982). Monophenolic compounds were eluted with 10 ml 72% aqueous methanol and equol and other diphenolic compounds with 5 ml 72% aqueous methanol saturated with CO_2.

Isolation of equol and daidzein glucuronides from human urine

Urine (20 ml) collected from a male subject after 2 days on a soya diet was extracted on a Sep-Pak C_{18} cartridge, filtered through a column bed (4 × 0·4 cm) of SP-LH-20 (H^+) as described above and fractionated on a column bed (6 × 0·4 cm) of TEAP-LH-20 in OH^- form. After elution of monoglucuronides of neutral compounds with 20 ml 0·8 M-acetic acid in 72% aqueous methanol, glucuronide conjugates possessing a free phenolic group were eluted with 15 ml 0·4 M-formic acid in 72% methanol (Sahlberg, Axelson, Collins & Sjövall, 1981). An aliquot of the material in this fraction was analysed by TLC as for soya. R_f values of the glucuronides of equol and daidzein were 0·28 and 0·23 respectively (for comparison, oestriol 16α-glucuronide had an R_f value of 0·29). The carboxyl group of the glucuronic acid was methylated with diazomethane and the methyl esters were dissolved in methanol and purified on a column bed (4 × 0·4 cm) of TEAP-LH-20. After washing with 5 ml

methanol the conjugated equol and daidzein derivatives (which possess a free phenolic group) were eluted with 8 ml CO_2-saturated methanol.

Preparation of derivatives for gas chromatography (GC)–mass spectrometry (MS)

Methyl esters

The dried extract was dissolved in 1 ml diethyl ether : methanol (9 : 1, v/v) and diazomethane (freshly prepared by the reaction between N-methyl-N-nitroso-toluenesulphonamide and aqueous KOH, in diethyl ether; Schlenk & Gellerman, 1960) was added to the sample through a stream of nitrogen. After 30 min in an ice bath, excess diazomethane and solvents were removed under a stream of nitrogen.

Trimethylsilyl (TMS) ethers

Trimethylsilyl ethers were prepared by addition of 100 µl pyridine : hexamethyldisilazane : trimethylchlorosilane (3 : 2 : 1, by vol.) and heating at 60 °C for 30 min. The reagents were removed under a stream of nitrogen and the derivatives dissolved in hexane.

Deuterium-labelled TMS ethers were prepared by reaction with 100 µl [$^2H_9$]trimethylchlorosilane (Merck Sharp & Dohme Canada Ltd, Montreal, Canada) : pyridine (1 : 18, v/v) at 20 °C for 30 min.

Gas chromatography and mass spectrometry

Gas chromatography

Gas chromatography was carried out on a Pye 104 gas chromatograph equipped with a flame ionization detector and housing a 20 m × 0·3 mm open-tubular glass capillary column coated with SE-30 (Orion Analytica, Espoo, Finland). Nitrogen was the carrier gas with an inlet pressure of 50 kPa, giving a flow rate of about 1 ml/min. The oven temperature was 250 °C.

Quantification of equol present in the hydrolysed urine fractions was obtained by comparison of its GC peak area with that given by a known amount of authentic equol, having a retention time as a TMS ether derivative of 0·45 relative to that of 5α-cholestane. The limit of detection corresponded to about 1 µg equol in a 24-h urine collection.

Gas chromatography–mass spectrometry

Gas chromatography–mass spectrometry was done on a modified LKB 9000 instrument housing an open-tubular glass capillary column (25 m × 0·3 mm) coated with SE-30, heated at 250 °C and connected to the ion source by a single stage adjustable jet separator (Axelson & Sjövall, 1977).

Derivatized conjugated compounds were analysed on a 1·5% SE-30 packed column (1 m × 3·4 mm) at 250 °C. Temperatures of the molecular separator and the ion source were 275 and 290 °C respectively; energy of bombarding electrons, 22·5 eV, ionizing current, 60 µA and accelerating voltage, 3·5 kV. Repetitive magnetic scanning (usually six to ten scans/min) over the range of mass/charge ratios (m/z) 0–800 daltons per unit electronic charge was initiated after a suitable delay from the time of sample injection. Methods for the computerized evaluation of the mass spectral data have been described (Axelson, Cronholm, Curstedt *et al.* 1974).

RESULTS

Dietary origin of equol

Since rats excrete equol in urine and bile (Axelson & Setchell, 1981) this animal was used as a model for man in the experiments screening for dietary precursors. Evidence that commercial pelleted food contains precursors of equol was obtained by changing a normal rat feed to that of a semisynthetic diet. A marked and rapid decrease in the urinary excretion of equol to less than 2 µg/24 h was followed by an increase after the diet reverted to pellets (Fig. 1). In the subsequent search for equol precursors, different food constituents and food extracts were added to this semisynthetic diet and the excretion of equol in urine was monitored. Of the variety of food-stuffs tested, which included soya flour, soya oil, wheat, rye, oat, millet, barley, buckwheat, corn, alfalfa, white beans and brown beans, soya flour provided the richest source of precursor(s) and resulted in the urinary excretion of about

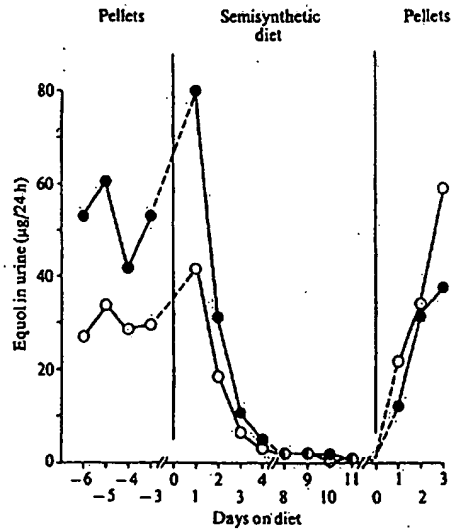

FIGURE 1. Daily urinary excretion of equol by two female rats fed commercial pelleted food or semisynthetic diet.

100 μg equol/g flour ingested (range 60–130 μg/g in five rats). The corresponding value for soya oil was only 5 μg/g. Little or no increase in the urinary excretion of equol (< 1 μg/g) was observed with the other food constituents tested. Commercial food pellets contain a proportion of soya cake sufficient to account for the urinary excretion of equol by rats.

Extending these studies to man, the urinary excretion of equol in two subjects (male and female), which is normally in the range of the classical oestrogens (Adlercreutz, Fotsis, Heikkinen *et al.* 1982; Axelson *et al.* 1982), increased 100- to 1000-fold to about 4–6 mg/24 h after ingestion of 40 g soya/day (Fig. 2). Thus the response to this diet was analogous to that observed in rats, indicating that the precursor–product relationships are similar in man and rats.

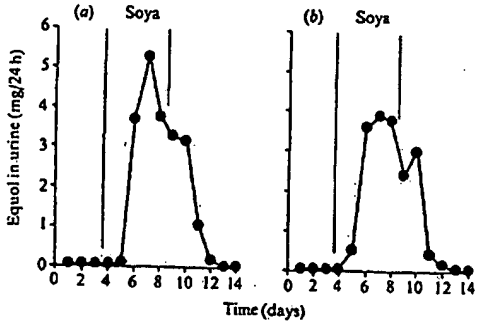

FIGURE 2. Daily urinary excretion of equol by (*a*) a man and (*b*) a woman before and after the addition of textured soya (40 g/day) to the normal diet.

Identification of daidzein, a precursor of equol in soya

Experiments to isolate the precursor(s) from soya flour showed that the compound(s) was extractable with 80% aqueous ethanol. After the fractionation of the extracted material into neutral and phenolic compounds on the ion exchanger TEAP-LH-20, approximately 90% of the precursor(s) was present in the phenolic fraction. Preparative TLC showed a major component of R_F 0·32. When this component was fed to rats it was readily converted to equol and quantitatively accounted for approximately 75% of the expected excretion of equol from the original amount of soya. The TMS ether of this compound, when analysed by GC–MS, did not show the molecular ion (M) at *m/z* 776 (Fig. 3) but ions at *m/z* 761 (M-15), 671 (M-(15+90)) and 581 (M-(15+2×90)), which are formed by loss of a methyl group from the molecule and subsequent loss of one and two derivatized hydroxyl groups respectively. The base peak at *m/z* 361 and the ions of *m/z* 271, 243, 217 and 204 are indicative of a TMS ether of a hexose structure (Laine & Elbein, 1971). In analogy with persilylated glucuronide conjugates (Spiegelhalder, Röhle, Siekmann & Breuer, 1976) the ion at *m/z* 450 indicated that the sugar residue was conjugated to an aromatic hydroxyl group. This ion arises from the glycone with a loss of a proton. Aromatic conjugation was further supported by the intense ion at *m/z* 398 (M-378), which is formed by deconjugation and transfer of a TMS group from the glycoside moiety to the aglycone (Billets, Lietman & Fenselau, 1973; Spiegelhalder *et al.* 1976). The ion at *m/z* 383 is formed by subsequent loss of a methyl group from the aglycone.

Treatment of this compound with β-glucosidase yielded a product (R_F value 0·83 compared with 0·92 for equol on TLC), the TMS ether derivative of which had an identical GC retention time on SE-30 (1·12 relative to 5α-cholestane) and mass spectrum as the persilylated authentic daidzein. The mass spectrum showed a molecular ion and base peak at *m/z* 398 (Fig. 3). The origin of ion *m/z* 355 is unknown, it may be due to loss of CH_3 (*m/z* 383) and CO (Budzikiewicz, Djerassi & Williams, 1964). The fragment ion at *m/z* 190 may represent $((CH_3)_3SiO\text{-}C_6H_4\text{-}C{\equiv}CH)^+$ in analogy with the fragmentation of equol (Axelson *et al.* 1982). These interpretations were supported by the analysis of the compound as a perdeuterated derivative.

The glycoside moiety of the daidzein conjugate occurring in soya was tentatively identified as a glucose residue. This is based on the following properties and evidence: hydrolysis with β-glucosidase, mobilities on the anion exchanger TEAP-LH-20, TLC and GC (retention time on SE-30 was 19 times that of the TMS ether of daidzein), the mass spectrum and the previous finding of daidzein glucoside in soya beans (Walz, 1931). Definite confirmation that daidzein is the major precursor of equol in commercial soya flour was obtained by feeding a rat the reference compound daidzein (acetate, 400 μg) in the semisynthetic diet, which yielded approximately 50 μg equol in the urine.

Identification of equol and daidzein glucuronides in human urine

In our earlier characterization of equol in urine (Axelson *et al.* 1982) it was tentatively identified as a glucuronide conjugate, consistent with the majority of endogenous urinary steroids. After repetitive scanning MS of the GC-effluent, the presence of equol and daidzein glucuronides was evident from the reconstructed chromatograms for diagnostically significant ions given by the methyl ester TMS ether derivative of the two compounds (Fig. 4). Peaks occurred at 14 and 32 min (retention time of 5α-cholestane was about

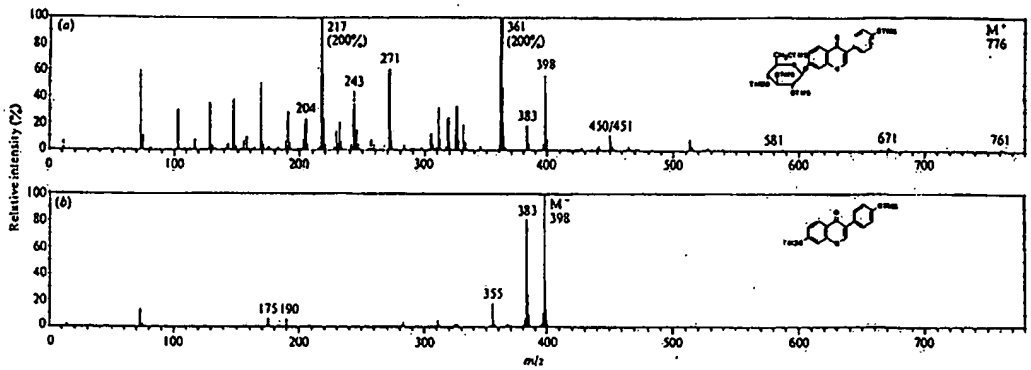

FIGURE 3. Electron impact ionization mass spectra of the trimethylsilyl (TMS) ethers of daidzein (4',7-dihydroxy-isoflavone) glucoside isolated from soya flour (*a*) before and (*b*) after treatment with β-glucosidase. M^+, molecular ion; *m/z*, mass/charge ratio in daltons per unit electronic charge.

l min) which represent intact glucuronides of equol and daidzein respectively. The complete mass spectrum of equol glucuronide eluted at 14 min is shown in Fig. 5. Consistent with the mass spectra of derivatives of glucuronide conjugates of oestrogens (Spiegelhalder *et al.* 1976) the relative intensities of the molecular ion (*m/z* 720) and the ions formed by losses of a methyl group (*m/z* 705) and TMS groups (*m/z* 615 and 525) are below 5% in this aromatic conjugate. The loss of 292 and 334 mass units from the molecular ion has previously been observed in mass spectra of phenolic glucuronide conjugates (Billets *et al.* 1973; Spiegelhalder *et al.* 1976; Axelson & Setchell, 1980); the latter fragmentation represents the loss of glucuronic acid with the corresponding transfer of a TMS group to equol. The ion at *m/z* 192 consists of a derivatized phenol group with a 2-carbon chain which is the base peak in the mass spectrum of unconjugated equol (Axelson *et al.* 1982). Ions at *m/z* 406, 407, 317 (base peak), 275, 217 and 204 are all typical of the fragmentation of the glucuronic acid (Billets *et al.* 1973; Spiegelhalder *et al.* 1976; Axelson & Setchell, 1980).

The glucuronide conjugate of daidzein was also identified in the same urine sample (Fig. 4). The mass spectral fragmentation pattern of the methyl ester TMS ether of the intact conjugate (Fig. 5) was similar

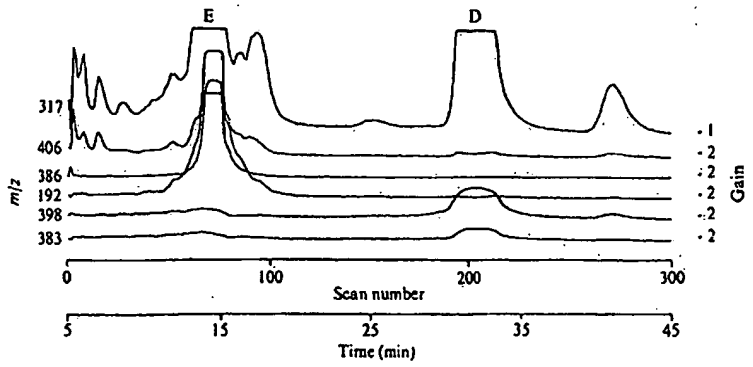

FIGURE 4. Gas chromatographic–mass spectrometric analysis of methyl ester trimethylsilyl ether derivatives of intact glucuronides of equol (E; 7-hydroxy-3-(4'-hydroxyphenyl)-chroman) and daidzein (D; 4',7-dihydroxy-isoflavone) isolated from human urine. Fragment ion current chromatograms of mass/charge ratio (*m/z*) 317 daltons per unit electronic charge and *m/z* 406 are representative of the glucuronyl moiety, those of *m/z* 386 and *m/z* 192 of equol and those of *m/z* 398 and *m/z* 383 of daidzein structure (see text). For purpose of illustration the intensities of *m/z* 317 were multiplied by a factor of 1, the other ions by a factor of 2.

to that of equol glucuronide. The loss of glucuronic acid in a rearrangement with the simultaneous transfer of a TMS group to the aglycone gives rise to the significant ion at *m/z* 398 (M-334) which is the molecular ion and base peak in the mass spectrum of the TMS ether of daidzein (Fig. 3).

These data firmly establish the occurrence of equol and daidzein in human urine as glucuronide conjugates. However, it is not possible to determine the position of conjugation by these methods, and the presence of two isomers of each compound cannot be excluded.

weeks the capacity to form equol decreased or disappeared in several rats. When pelleted food was again given, the capacity was partly regained. A variable yield of urinary equol has also been observed in man after ingestion of soya (Setchell *et al.* 1984). Conjugation of equol and daidzein with glucuronic acid most likely occurs in the liver as is the case with most endogenous oestrogens.

Although daidzein was the only equol precursor identified from soya, other precursors may exist in plants and foods. Isoflavones such as formononetin (7-hydroxy-4'-methoxyisoflavone), biochanin A (5,7-di-

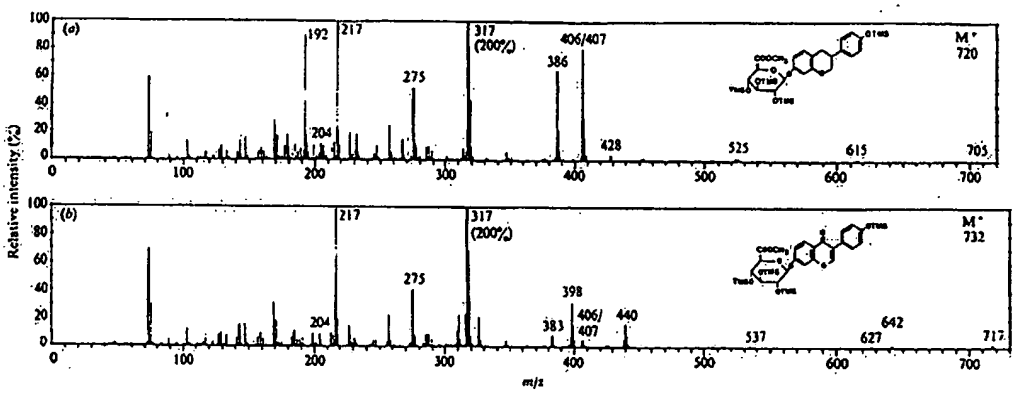

FIGURE 5. Mass spectra of the methyl ester trimethylsilyl (TMS) ether derivatives of the glucuronides of (*a*) equol (7-hydroxy-3-(4'-hydroxyphenyl)-chroman) and (*b*) daidzein (4',7-dihydroxy-isoflavone) isolated from human urine. The origin of ions is given in the text. M^+, molecular ion; *m/z*, mass/charge ratio in daltons per unit electronic charge.

DISCUSSION

As shown here, and in a subsequent study (Setchell, Borriello, Hulme *et al.* 1984), soya meal is a major dietary source of urinary equol in man. Equol itself was not detected in soya but was shown to be formed from the glucoside of daidzein. Glucosidases are known to be present in intestinal bacteria (Drasar & Hill, 1974), which have also been shown to carry out the reduction and deoxygenation reactions required for conversion of daidzein to equol in animals (Batterham *et al.* 1965; Nilsson *et al.* 1967; Batterham *et al.* 1971) and recently in man (Setchell *et al.* 1984). Germfree rats, however, do not excrete equol when given commercial pelleted food (Axelson & Setchell, 1981). These results strongly suggest that also in man equol is formed in the gastrointestinal tract as a result of the bacterial degradation of daidzein. Thus, the rate of formation of equol from daidzein is conceivably influenced by the composition of the microflora, the intestinal transit time and the redox level in the large intestine. These conditions are affected by the diet, and when rats were fed the semisynthetic diet for several

hydroxy-4'-methoxyisoflavone) and genistein (4'-5,7-trihydroxyisoflavone) are all potential precursors of equol in animals (Cayen, Carter & Common, 1964; Batterham *et al.* 1965, 1971; Nilsson *et al.* 1967; Shutt & Braden, 1968; Tang & Common, 1968; Batterham *et al.* 1971). Soya beans can contain an abundance of phyto-oestrogens (Walz, 1931; Walter, 1941; Naim, Gestetner, Kirson *et al.* 1973; Lookhart, Jones & Finney, 1978), particularly genistein and daidzein, which have been ascribed to cause uterotrophic effects in laboratory mice given soya bean meal (Carter, Smart & Matrone, 1953; Cheng, Story, Yoder *et al.* 1953) or commercial pelleted food (Drane, Patterson, Roberts & Saba, 1975, 1980). Our observations here and earlier that equol is the major phenolic compound found in urine, blood and bile of rats maintained on this diet (Axelson & Setchell, 1981) suggest that the oestrogenic effects are more likely to be induced *in vivo* by equol than by genistein and/or daidzein. Genistein glucoside was not detected in the commercial soya flour used in the present study. Whether this is due to variations in the composition of isoflavones between different species of soya beans or due to elimination of

genistein during the manufacture of the flour is not known.

The widespread use of soya beans as a protein food source makes it important to determine possible physiological effects of equol in man. The 'contraceptive' effect in animals suggests to us that it may be of interest to investigate the dietary habits and urinary excretion of equol in women with unexplained infertility or disorders of the menstrual cycle.

In addition, whether the presence of phytooestrogens and related oestrogenic compounds in common food-stuffs affect the development and/or treatment of hormone-dependent tumours should also be considered.

ACKNOWLEDGEMENTS

The excellent technical assistance of Ms B. Mörk, E. Conway and Mr M. Corrie is gratefully acknowledged. Professor D. N. Kirk kindly provided samples of equol and daidzein. The work was supported by grants from the Swedish Medical Research Council (grants No. 03X-219 and 16X-206) and Karolinska Institute.

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COMMENTARY

The Role of Soy Products in Reducing Risk of Cancer[1]

Mark Messina, Stephen Barnes*

Since the initial recognition that diet plays a role in the etiology of certain cancers, particularly cancers of the breast and colon, considerable progress has been made in identifying dietary patterns associated with cancer risk. There is general agreement that a high-fat, low-fiber diet, like that consumed by much of the industrialized world, increases cancer risk and that plant-based diets, rich in whole grains, legumes, and fruits and vegetables, are protective. It has been, however, considerably more difficult to identify specific foods, types of food, or components of foods that influence cancer risk.

The recent workshop on The Role of Soy Products in Cancer Prevention, sponsored by the National Cancer Institute, had two objectives: 1) to evaluate the role of soybeans, food products derived from soybeans, and specific components of soybeans in the dietary prevention of cancer and 2) to recommend research initiatives and approaches for further studies of the effect of soy intake on human cancer risk. The meeting was chaired by Stephen Barnes and organized by Mark Messina.

Isoflavones in Cancer Prevention

Kenneth Setchell, Donna Baird, and Barnes discussed the potential role of isoflavones in the prevention of cancer. Setchell reviewed the history of phytoestrogens (*1*), noting that plants were first observed to induce estrus in animals in 1926. Over 300 plants are now known to possess estrogenic activity (*2,3*). In 1946, the infertility observed in Australian sheep that grazed on a certain type of subterranean clover was attributed to the high isoflavone content of this plant (*4*). Ruminal bacteria in these animals convert plant isoflavones into the mammalian isoflavone equol, which, following absorption, may suppress the pituitary gonadotropic axis. Equol, a weak estrogen possessing about 0.2% of the biological activity of estradiol, was first identified in human urine in 1982 by Setchell et al (*5,6*). Setchell's further interest in the potent estrogenic effects of soybean isoflavones was stimulated coincidentally. He discovered that the soy component of diets fed to captive cheetahs, which was added for economic reasons, was responsible for the severe breeding problems in these animals (*6,7*).

Setchell noted that isoflavone metabolism has been studied in humans, although only superficially. In one study, subjects fed 40 g of soy daily were found to have urinary levels of equol as much as 1000-fold higher than baseline values (*8,9*). The low levels of urinary equol in two of the six subjects in this study indicate that the intestinal microflora (*10*) participate in isoflavone metabolism and that isoflavones undergo enterohepatic circulation (*10*). Improved analytical methods (*11,12*) have led to the realization that equol represents only a small fraction of the total amount of isoflavone in urine and that conjugates of the soybean isoflavones daidzein and genistein are the major forms present. The high levels of isoflavone in urine in subjects fed soy suggest that these compounds are likely to elicit a biological response (*13*).

Setchell concluded his presentation with a reminder (*a*) that all weak estrogens can also have antiestrogenic activity; (*b*) that tamoxifen, which has been used therapeutically for breast cancer, is structurally related to some of the phytoestrogens; and (*c*) that vegetarians, who may have a lower risk of certain cancers, excrete higher levels of phytoestrogens. These findings have led to collaborative studies by Barnes, Setchell, and associates (*14*), who used an animal model designed to test the hypothesis that phytoestrogens have a role in reduction of breast cancer risk.

[1]Report of a workshop held June 26-27, 1990, at the Guest Quarters Hotel in Bethesda, Md. Workshop members were Donna Baird, National Institute of Environmental Health Sciences, Research Triangle Park, NC; Stephen Barnes, University of Alabama at Birmingham, Birmingham, Ala; David L. Brandon, Western Regional Research Center, United States Department of Agriculture, Albany, Calif; James A. Duke, Agricultural Research Service, United States Department of Agriculture, Beltsville, Md; Ernst Graf, The Pillsbury Co, Minneapolis, Minn; Ann R. Kennedy, University of Pennsylvania Medical School, Philadelphia; Renee M. Kosslak, Iowa State University, Ames; Irvin E. Liener, University of Minnesota, St. Paul; Mark Messina, National Cancer Institute, Bethesda, Md; Frank L. Meyskens, University of California, Irvine, Calif; A. Venket Rao, University of Toronto, Ontario, Canada; Kenneth D. R. Setchell, Children's Hospital, Cincinnati, Ohio; Bernie P. Szuhaj, Central Soya, Fort Wayne, Ind.

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Barnes began by observing that Oriental women, who have low incidence rates of breast cancer (*15*), consume larger amounts of soy products than do most American women. However, although fertility and reproduction in animals are adversely affected by ingestion of plant isoflavones, the amount of isoflavones in soy products consumed by Oriental women does not appear to affect their reproductive capacity.

Barnes discussed the recent animal study conducted in collaboration with Setchell and other investigators (*14*). In that study, consumption of soybeans significantly decreased chemically induced rodent mammary cancer. Rats were fed one of four soy products: powdered soybean chips consisting of unpurified soybeans, both raw and autoclaved; soy protein isolate composed of 91% protein; soy molasses, a concentrate of the aqueous alcohol extract of soy flour; and aqueous alcohol-extracted soy protein concentrate. All diets were isocaloric and isonitrogenous and produced similar weight gain among the animal groups throughout the study.

The first three products, all of which are rich in isoflavones, inhibited mammary tumorigenesis induced by 7,12-dimethylbenz[*a*]anthracene or methylnitrosourea, while the aqueous alcohol-extracted soy protein concentrate, which had a low content of isoflavones, did not. Whether the soybeans were raw or cooked made no difference in the degree of inhibition of mammary cancer; cooked soybeans were shown to be devoid of protease inhibitor activity.

Barnes said the reduction in levels of mammary tumor estrogen receptors induced by the powdered soybean chips paralleled the inhibition of tumorigenesis and supported the hypothesis that the isoflavones exerted an antiestrogenic effect. Interestingly, however, this was not the case for the soy protein isolate. The decrease in levels of mammary tumor estrogen receptors was smaller than predicted from the degree of tumor inhibition, he said, suggesting that the antiestrogenic effect of isoflavones may not be the primary mechanism responsible for inhibition of tumorigenesis. Therefore, Barnes concluded, the anticarcinogenic activity of isoflavones may not be limited to tumors containing a functional steroid receptor system. Alternative mechanisms of action may include inhibition of the activity of tyrosine protein kinases (eg, epidermal growth factor receptor tyrosine kinase) (*16*), DNA topoisomerase II (*17*), and ribosomal S6 kinase (*18*), as well as induction of specific cytochrome P450s (*19*).

Baird, before describing her recent study of the effects of feeding soy to postmenopausal women (manuscript in preparation), cited the concern of the National Institute of Environmental Health Sciences about the possible effects of low-level environmental estrogens on health. In her study, changes in estrogenic activity in postmenopausal women consuming soy over a 4-week period were examined. Soy was chosen for this study because of its high estrogenic activity (*20,21*), its increasing use in the United States, and the variety of products derived from soy and because soy consumption would not adversely affect nutritional status (*22*). Subjects consumed daily one main soy dish (½ cup of soybeans or 38 g of texturized vegetable protein) and two soy snacks—either soy chips (a roasted soybean product) or a spread for crackers made from the whole soybean. The estimated isoflavone content was about 200 mg/day, the equivalent of about 0.3 mg/day of conjugated steroidal estrogen, assuming that the estrogenic activity of phytoestrogens is about 0.1% that of conjugated estrogen.

Baird said preliminary findings indicate that, compared with control subjects, significantly more women fed soy exhibited an estrogenic response, as demonstrated by an increase in the number of superficial cells of the vaginal epithelium. She remarked that postmenopausal women were chosen for this study because of the decision to examine the estrogenic rather than the antiestrogenic effects of plant phytoestrogens. In premenopausal women with relatively high estrogen levels, the antiestrogenic effects of soybeans may have been observed.

Protease Inhibitors

Ann Kennedy, David Brandon, and Irvin Liener focused their attention on the soybean protease inhibitors. Kennedy reviewed her work, as well as that of others, in the field of protease inhibitors and cancer prevention. She noted that the soybean-derived Bowman-Birk protease inhibitor (BBI) either inhibits or prevents development of experimentally induced colon (*23*), oral (*24*), lung (*25*), liver (*23*), and esophageal cancers (von Hofe E, Newberne P, Kennedy A: unpublished observations). Protease inhibitors, at the levels used in these studies, do not adversely affect animal growth. Kennedy noted that the anticarcinogenic effect of the BBI is thought to stem from its ability to inhibit chymotrypsin activity (*26*), but results also suggest an important role for trypsin inhibition in suppression of the promotional stage of carcinogenesis (*27*). She said in vitro work indicates that protease inhibitors prevent conversion of normal cells to the malignant state even at very late stages in carcinogenesis but that they have no effect on cancerous cells (*28*). Protease inhibitors are unique in that they cause an irreversible suppressive effect on the carcinogenic process. They have also been shown to suppress oncogene expression and to inhibit carcinogen-induced protease activity (*29*).

Kennedy said recent data suggest that the antigrowth effects of raw soybeans commonly attributed to protease inhibitors may actually be due to an unidentified factor(s) (*30*). Furthermore, in human populations consuming soybeans, the connection between pancreatic enlargement and protease inhibitors observed in animals has not been seen. In fact, incidence of pancreatic cancer is decreased in these groups (*31*). Kennedy noted that in vitro comparisons of the pure BBI with an extract of soybeans containing BBI indicate that the activity of the soybean extract could be directly attributable to BBI (*26*). However, she said an in vivo study suggests that the extract may contain an additional anticarcinogenic agent working in conjunction with the BBI (*26*). The extract contains approximately 50% protease inhibitor; the remaining content is unknown, but it may include isoflavones as well as other potential anticarcinogens. Kennedy commented that the lowest effective dietary levels of protease inhibitors used in these animal studies (0.1%) could be achieved by humans by modifying the diet to include soy products.

Brandon discussed the measurement of protease inhibitors in soybeans and soy products, noting the concern of the Agricultural Research Service of the United States Department of Agriculture (USDA) over the possible adverse effects of

protease inhibitor intake in humans, particularly in infants (*32*). Enzyme-linked immunosorbent assays (ELISA), using monoclonal antibodies, have been developed for the measurement of two different protease inhibitors found in soybeans—BBI and Kunitz trypsin inhibitor (KTI) (*33,34*). These procedures are suitable for quantifying residual protease inhibitor levels in foods. A variety of processed soy products, a series of soybean flours derived from seeds in the USDA Soybean Germplasm Collection, and the soybean isoline L81-4590 (lacking KTI) (*35*) have been analyzed. Brandon noted that an important observation from the ELISA analysis of heat-treated soy flours derived from the isoline was that KTI, not BBI, is responsible for the heat-stable activity of commercial soy flour that inhibits trypsin activity (*36,37*). The microenvironment of the soy flour appears to promote heat inactivation of BBI to a greater extent than it affects KTI. This finding contrasts with the results of work showing that BBI is relatively heat stable in the pure form (*38*). Moisture, fat content, the presence of agents that influence changes in disulfide bonds, and interactions with other constituents, such as carbohydrates, appear to influence the denaturation of inhibitors (*39*).

Brandon said analysis of infant formula has revealed that active KTI and BBI, when measured on the basis of weight per gram of protein, are reduced to about 0.1% of their levels in raw soy (*40*). An infant on a diet consisting exclusively of soy formula would consume about 10 mg of active KTI plus BBI per day. In toasted (autoclaved) soy flour, 20%-30% of the KTI activity remains, while all of the BBI is inactivated. Analysis of tofu (soybean curd) has revealed that the protease inhibitor content varied significantly among the samples, from 4 to 30 μg of BBI and from 5 to 16 μg of KTI per gram of product. The protease inhibitor content of several soy protein isolates also varied, as much as 20-fold. Not unexpectedly, there was also a wide variation in the protease inhibitor content among varieties of soybeans. Brandon suggested that food-processing strategies could be combined with genetic approaches to optimize the protease inhibitor content of soy products.

Liener reviewed research on the potential adverse effects of consuming protease inhibitors, first noting that most work has been done with small experimental animals (*41*). Consumption of raw soybeans has two major effects: growth inhibition and pancreatic enlargement. Rats consuming raw soy flour for extended periods develop adenomatous nodules involving acinar cells of the pancreas (*42*). Additionally, raw soy flour consumption potentiates the effect of pancreatic carcinogens (*43*). In a study by Liener et al (*44*), heat treatment of raw soybeans almost completely eliminated this potentiation, while the addition of protease inhibitors to the heated product restored most of the pancreatic enlargement observed with raw soy, suggesting that protease inhibitors are at least partly responsible for pancreatic enlargement.

Liener noted that the varied response to raw soy flour among species is particularly important. Rats, mice, chickens, hamsters, and young, growing guinea pigs all exhibit pancreatic enlargement in response to protease inhibitors, while dogs, pigs, calves, and monkeys do not (*45*). Growth inhibition induced by soybean products is thought to result from a deficiency of the sulfur-containing amino acids caused by the dramatic increases in fecal levels of endogenous protease enzymes, particularly trypsin and chymotrypsin, two enzymes that are rich in these amino acids (*46*).

Commenting that pancreatic enlargement apparently stems from elevated serum levels of the hormone cholecystokinin, Liener commented that pancreatic enzyme secretion is inversely related to the level of trypsin in the intestine, a process regulated by cholecystokinin. This hormone stimulates the pancreas to produce trypsinogen, but because the protease inhibitors combine with trypsin, the suppressive effect of trypsin on intestinal release of cholecystokinin is eliminated (*47*).

Liener raised the question: Can the effects of protease inhibitors in small animals be extrapolated to humans? A negative feedback system in humans has been observed (*48*). Directly supplying BBI or raw soy flour to the duodenum causes an increase in secretion of pancreatic enzymes (*48*) and in blood levels of cholecystokinin (*49*). (BBI, in contrast to KTI, survives in gastric juice.) Despite these observations, he said, it is not possible at this time to accurately assess the health consequences of consuming processed soy products.

Phytosterols and Saponins

A. Venket Rao presented evidence for reduction of colon cancer risk by phytosterols and saponins. Both substances are common constituents of plants, but the concentration in soybeans is particularly high. Phytosterols are structurally similar to the animal sterol cholesterol. They inhibit cholesterol absorption and are almost quantitatively recoverable in fecal material, indicating that very little intestinal absorption occurs (*50*). Soybeans are a major contributor of phytosterols to the diet, particularly β-sitosterol (90 mg/100 g edible portion of the soybean) (*51*). Soybean oil is potentially an important source of phytosterols, but upon refinement and hydrogenation, phytosterol levels are reduced from 315 mg to 217 mg and 132 mg, respectively, per 100 g of oil (*51*). Dietary phytosterol intake among populations differs dramatically; the typical western diet contains about 80 mg/day, while Japanese and vegetarian diets provide about 400 and 345 mg/day, respectively (*52,53*).

In addition to the phytosterols, whole soybeans contain significant amounts of saponins, about 5% of dry weight (*54*), while tofu contains approximately half that much. Saponins are amphophilic compounds having surfactant properties and, like phytosterols, bind to cholesterol and bile acids.

Rao said that while nutritional interest in both phytosterols and saponins has focused on their cholesterol-lowering properties, some data suggest that these compounds may be anticarcinogens. In rats, β-sitosterol-supplemented diets (0.2% by weight) inhibit chemically induced colon cancer (*55*), and phytosterols reduce, in a dose-dependent fashion, cholic acid-induced colon cell proliferation and mitotic activity (*56*). Diets containing phytosterols at 1% by weight are well tolerated by experimental animals (*57*). Dietary saponins from soybeans and other sources have been shown to enhance immunity (*58,59*), are cytotoxic to Sarcoma 37 cells (*60*), inhibit DNA synthesis in tumor cells (*61*), decrease the growth of human epidermoid carcinoma cells (*62*) and human cervical carcinoma cells (*63*), and inhibit Epstein-Barr virus genome expression (*64*). Saponin-sup-

plemented diets (1% by weight), as is the case for the phytosterols, normalize abnormal colonic cell proliferative activity induced by carcinogens (Rao AV: unpublished observations).

Inositol Hexaphosphate

Ernst Graf discussed the rationale for the hypothesis in which inositol-1,2,3,4,5,6-hexaphosphate (IP_6), not fiber, is postulated to be responsible for the inverse correlation between the incidence of colon cancer and the consumption of fiber-rich foods (*65*). When the IP_6 content of cereals, fruits, and vegetables is considered, the international data suggest that there is a greater negative correlation between IP_6 and colon cancer incidence than between fiber and colon cancer incidence. IP_6 is found in a variety of plant foods, particularly cereals, but soybeans are an especially rich source, containing about 1.4% on a dry-weight basis (*66*).

Graf noted that most nutritional interest thus far has focused on the inhibitory effect of IP_6 on mineral absorption. IP_6 forms tight chelates with a variety of polyvalent metals such as calcium, zinc, and iron (*66*). However, he said, the ability to bind metal ions, particularly iron, may provide the basis for the anticarcinogenic effects of this compound. Graf commented that iron may be a key factor, via the Haber-Weiss reaction, in the production of hydroxyl radicals, which are postulated to play a role in the etiology of some cancers (*67*). IP_6 has been shown to limit the oxidant reactivity of transition metals (*66*), to inhibit lipid peroxidation (*67*), and to inhibit experimentally induced colon cancer (*68-73*). It has also been suggested that IP_6, through absorption following dephosphorylation to IP_3, could be an important second messenger involved in the regulation of cell differentiation (*73*).

Phytochemical Variation

James Duke discussed phytochemical variation in soybeans. Duke started by noting that there are over 10 000 named or numbered varieties of the common soybean *Glycine max L.* In these varieties, as one might expect, lies tremendous chemical variation. The genus *Glycine* was originally applied to a distant relative, now known as *Apios americana*, which is an edible root with more protein than is found in potato (*74*).

The isoflavone content of soybeans varies tremendously according to the plant part, variety, year harvested, and geographic location (*75*). Soybean hulls contain only relatively minor amounts of isoflavones, the majority of which occur in the hypocotyl, although one common isoflavone, genistein, is found primarily in the cotyledon (*75*). Equally significant are the reported differences in isoflavone content according to the varieties of soybeans and the year harvested. One study (*75*) reported a threefold variation in total isoflavone content among four varieties of soybeans, while a 30% variation was noted in a single variety of soybeans over a 4-year period. The content of individual isoflavones varied as much as 50%. Not surprisingly, location influences isoflavone content, even within fairly close geographical areas.

Duke noted that chemical variation is not limited to the isoflavones. In some instances as much as a fivefold variation was found among different phenolic acids in soybeans, many of which have also been investigated as potential anticarcinogens.

Isoflavones in Plant Physiology

Renee Kosslak described the role of isoflavones in defense strategies utilized by plants. Plants produce a wide range of products or secondary metabolites thought to enhance their survival (*76*). The isoflavones daidzein and genistein are the major inducers of the nodulation genes in *Bradyrhizobium* bacteria, which form nodules on soybeans (*77*).

The genetic regulation of isoflavone synthesis in plants is not well understood, in part because of the limited number of appropriate mutants affecting this pathway (*78,79*). In soybeans, near-isogenic lines that differ in their root fluorescence are being examined to determine whether they are active in genetic regulation of isoflavone synthesis (*80*). (These differences in root fluorescence in soybeans were first observed in 1934.) There are five loci that affect root fluorescence (*80*), and although specific substances responsible for this property have not been identified, isoflavones are thought to be involved. Preliminary data indicate that the levels of daidzein, genistein, and coumestrol, which is also a phytoestrogen, were either reduced or absent in root extracts from three of the nonfluorescent isolines tested (Kosslak R: unpublished observations).

Kosslak suggested that if future research implicates isoflavones and/or phytoestrogens as important dietary factors in cancer prevention and if the demand for soybean specialty products materializes, it may be possible to manipulate levels of these compounds in soybeans, using root fluorescence as a marker.

Soybean Processing

Bernie Szuhaj briefly discussed soybean processing procedures (*81-83*). Solvent extraction is the primary method of producing soybean products today. Soybeans entering the plant are first cleaned, cracked, and dehulled. Then moisture is added so they can be "flaked," leaving a product that is 3% hypocotyl, 89% cotyledon, and 8% hulls. The oil is removed from the flakes by hexane, producing defatted flakes and soybean oil. From the defatted flakes come a variety of products with a protein content, on a dry-weight basis, that ranges from about 50% for soy flour and grits to about 60%-70% for protein concentrates and about 90% for protein isolates. The primary difference between soy protein concentrates and isolates is the larger percentage of carbohydrate in the soy protein concentrates. Many commercial doughnuts contain soy flour, and, in Europe and Asia, there is particular interest in the use of full-fat soy flours for baking.

Szuhaj noted that most soybean production today goes into animal feed, while the soy protein concentrates and isolates are marketed primarily for their multifunctional properties, such as emulsifying, gelling, fat-binding, texturizing, and dough forming. Soy products play a major role in the food chain. They are added to a wide variety of foods, from cereals to chili. Some

meat products, such as ground beef, contain up to 25% soy. These products have been used in the Armed Forces' canteens since 1983 and in the federal school lunch program.

Discussion

This workshop had two objectives: *1*) to evaluate the relationship between the risk of certain cancers and consumption of soybeans, products derived from soybeans, and/or specific components of soybeans and *2*) to recommend research initiatives aimed at clarifying this relationship. The consensus of the meeting was that there are sufficient data to justify studying the impact of soybean intake on cancer risk in humans.

There were three workshop recommendations. First, future dietary studies involving soybeans should be carried out using soy products rather than isolated compounds, since soybeans appear to contain several potential anticarcinogens. Additionally, because components of food interact, both negatively and positively, with each other, the potential benefit of soy products cannot be accurately predicted solely on the basis of the effects of individual soybean components. This does not, however, prohibit future use of isolated soybean components as chemopreventive agents in clinical trials. Second, standardized and improved analytical methods are needed so that the contents of all soy-based materials employed in soybean research, whether soybean fractions or soy products, can be accurately described. This methodology will allow for valid comparisons among studies. Third, basic research on the absorption, metabolism, and physiology of potential anticarcinogens in humans should be conducted. This research will likely help to determine the clinical relevancy of these compounds and to provide a basis for selecting specific soy products for use in future dietary studies.

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Use of a Mammalian Cell Culture Benzo(*a*)pyrene Metabolism Assay for the Detection of Potential Anticarcinogens from Natural Products: Inhibition of Metabolism by Biochanin A, an Isoflavone from *Trifolium pratense* L.[1]

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ABSTRACT

Based on the epidemiological evidence for a relationship between consumption of certain foods and decreased cancer incidence in humans, an assay was developed to screen and fractionate plant extracts for chemopreventive potential. This assay measures effects on the metabolism of [^{3}H]benzo(*a*)pyrene [B(a)P] in hamster embryo cell cultures. Screening of several plant extracts has generated a number of activity leads. The 95% ethyl alcohol extract of one of these actives, *Trifolium pratense* L. Leguminosae, red clover, significantly inhibited the metabolism of B(a)P and decreased the level of binding of B(a)P to DNA by 30 to 40%. Using activity-directed fractionation by solvent partitioning and then silica gel chromatography, a major active compound was isolated and identified as the isoflavone, biochanin A. The pure compound decreased the metabolism of B(a)P by 54% in comparison to control cultures and decreased B(a)P-DNA binding by 37 to 50% at a dose of 25 μg/ml. These studies demonstrate that the hydrocarbon metabolism assay can detect and guide the fractionation of potential anticarcinogens from plants. The ability of the isoflavone biochanin A to inhibit carcinogen activation in cells in culture suggests that *in vivo* studies of this compound as a potential chemopreventive agent are warranted.

INTRODUCTION

Humans are exposed to numerous carcinogens and mutagens daily, some avoidable (such as cigarette smoking) and some virtually unavoidable (diet, environmental pollution, oxygen radicals). The diet has been shown to have a profound effect on the incidence and location of various human cancers worldwide (1, 2), and epidemiological studies suggest that certain dietary components may help to prevent cancer induction. This prophylaxis has been termed cancer chemoprevention. Wattenberg (3) has demonstrated that such agents may inhibit cancer induction by a number of mechanisms. One of the more common mechanisms is through inducing alterations in the enzymatic activation or detoxification of carcinogens.

Although many biological assays have been used to examine the chemopreventive potential of various chemicals, there have been relatively few studies using activity-directed fractionation to isolate active compounds from plants. In addition, it is impractical to use *in vivo* models to guide these procedures. Loub *et al.* (4) used an activity-directed fractionation procedure based upon induction of aryl hydrocarbon hydroxylase activity in the liver and intestinal mucosa of Sprague-Dawley rats to isolate and identify several indoles from cruciferous vegetables. Kaweol and caffestol palmitates were isolated from green coffee beans (5) based upon an assay that measured the increase in glutathione S-transferase activity in liver and intestinal mucosa of mice. Practical assays for activity-directed fractionation of active plants must be rapid, sensitive, convenient, and capable of detecting alterations in carcinogen metabolism. In this paper, we describe the development and application of an assay that measures effects on the metabolism of [^{3}H]benzo(*a*)pyrene, a widespread environmental carcinogen, in early passage cultures of Syrian hamster embryo cells (6). The chemical and analytical procedures developed for activity-directed fractionation of antineoplastic compounds from plants (7, 8) were adapted to the isolation and identification of potential anticarcinogens from food and food plants, such as red clover extracts, which significantly inhibited the metabolism of benzo(*a*)pyrene and binding of B(a)P[3] metabolites to DNA.

MATERIALS AND METHODS

Spectroscopy and Chromatography. ^{1}H NMR in deuteriochloroform was performed using a Varian XL-200, and ^{13}C NMR in deuteriochloroform was measured on a Chemagnetics A-200 spectrometer. EI and CI mass spectra were obtained on a Finnigan 4023 quadrupole mass spectrometer. High-resolution mass spectra were recorded on a Kratos MS 50. The IR spectrum was performed on a Beckman IR-33 using a KBr pellet. UV spectra were measured on a Beckman DU-7 in methyl alcohol using sodium methoxide, $AlCl_3$, HCl, and sodium acetate as UV shift reagents.

For flash column chromatography EM 9385 Silica Gel 60 was used for the adsorbent. Radial chromatography was performed on a Chromatotron Model 7924 using a 1-, 2-, or 4-mm rotor with EM 7749 Silica Gel 60 PF 254 as adsorbent. TLC plates were Merck 5714 Silica Gel 60 F_{254}.

Cell Culture Toxicity Assay. Hamster embryo cell cultures were prepared and grown as described previously (6). Tertiary cultures were plated in 60-mm plastic dishes (Falcon) (5×10^5 cells), and 24 h later the test compound was added at 10-fold dilutions from 500 μg/ml of medium to 0.05 μg/ml for 24 h. At that time the cultures which were approximately 70% confluent were examined microscopically and subjectively evaluated for the percentage of the cells dividing and the cell density. The highest noninhibitory dose was selected for metabolism studies.

B(a)P Metabolism Assay. Tertiary hamster embryo cell cultures (10^6 cells per 25-cm^2 flask, 3 flasks per group) were plated in 8 ml of medium containing 10% calf serum and refed with 8 ml of fresh medium after 48 h. Seventy-two h after plating, the cultures were treated with the test compound in DMSO or DMSO as a control and 30 min later [^{3}H]-B(a)P (1 μg/ml; specific radioactivity, 0.25 Ci/mmol) was added. Twenty-four h later medium was removed and stored at −20°C. Aliquots (0.2 ml) were extracted by a two-stage chloroform:methanol:water procedure (6, 9). The assay uses initial mixing with a vortex mixer in a single-phase system of chloroform:methanol:water (including the medium) (1:2:0.8) to ensure complete extraction of the lipophilic hydrocarbon and its metabolites followed by addition of 1 ml of chloroform

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[3] The abbreviations used are: B(a)P, benzo(*a*)pyrene; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; MS, mass spectrum; BHA, butylated hydroxyanisole; DMSO, dimethyl sulfoxide; B(a)PDE, 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene; dGuo, deoxyguanosine.

and 1 ml of water and mixing with a vortex mixer. After centrifugation for 10 min, the aqueous phase was removed and extracted with 2.0 ml of chloroform to ensure complete extraction. The chloroform extracts were then pooled, and the radioactivity in the organic and aqueous-methanol phases was measured by liquid scintillation counting of 0.1-ml aliquots. This extraction procedure results in recovery of unmetabolized B(a)P and Phase I metabolites (dihydrodiols, quinones, and phenols) in the chloroform phase. The water-soluble metabolites including glucuronides and glutathione conjugates and multiple oxidation products are retained in the aqueous-methanol phase. Since the large majority of the metabolites formed from B(a)P in hamster embryo cells are water soluble (usually greater than 80%) (6), this assay provides a rapid measure of B(a)P metabolism.

BHA, a known inhibitor of carcinogenesis and B(a)P metabolism (10), was used to treat a positive control group in all assays at a concentration of 50 μg/ml of medium. The highest nontoxic dose of BHA was selected from multiple experiments using different hamster embryo cell preparations. Doses of 75, 65, 50, and 5 μg/ml of medium were tested, and the results show that 75 μg/ml were toxic and 65 μg/ml exhibited borderline toxicity, while 50 μg/ml showed a significant inhibition of B(a)P metabolism with no cell toxicity. The lowest dose, 5 μg/ml, produced no significant inhibition of B(a)P metabolism. Using BHA as a positive control gave us an indication of the health and viability of the cells in the culture assay for that particular experiment and helped eliminate false negatives.

Analysis of B(a)P Metabolites. The B(a)P metabolites in the organic phase were analyzed by HPLC on an Ultrasphere C_{18} column (25 cm × 4.6 mm) eluted with a methanol:water gradient as described previously (6). UV absorbing standards of authentic B(a)P metabolites (Chemical Repository, Division of Cancer Etiology, National Cancer Institute) were included in each HPLC analysis. The radioactivity was monitored with a Flo-one β flow monitor set to update every 30 s.

Binding of B(a)P to DNA. Tertiary hamster embryo cell cultures (5×10^7 cells) were plated in 175-cm^2 flasks containing 50 ml of minimal essential medium with 10% fetal bovine serum. After 2 days the cultures were refed with fresh medium and 24 h later with the test compound, or extract in DMSO was added. Five to 10 min later the cultures were treated with [^{3}H]B(a)P (1 μg/ml of medium, 0.5 mCi/flask). After 24 h of incubation at 37°C the cells were harvested, and DNA was isolated as described previously (11). The radioactivity in an aliquot was measured by liquid scintillation counting, the amount of DNA was determined by A_{260}, and these values were used to calculate the level of B(a)P metabolites bound to DNA.

After enzymatic degradation of the DNA to deoxyribonucleosides, the B(a)P:deoxyribonucleoside adducts were isolated by chromatography on Sep-Pak C_{18} cartridges and analyzed by HPLC on a 25-cm × 4.6-mm Ultrasphere C_8 reversed-phase column (11). The column was eluted at a flow rate of 1.0 ml/min with methanol:water (46:54) for 34 min, a linear gradient for 10 min (46:54 to 55:45) and at 55:45 for 24 min. Fifteen 1.0-ml fractions followed by 165 fractions (0.3 ml) were analyzed by scintillation counting.

Plant Extraction. Leaves, stems, and flowers of *Trifolium pratense* L. (red clover) were collected. A voucher specimen is on deposit in the biology herbarium of the Department of Biology, Purdue University. The fresh plant (918 g) was ground with 2 liters of 95% ethyl alcohol in a commercial size Waring blender for 5 min. The blended material was then allowed to stand for 30 min to complete the extraction. The material was then filtered through a Büchner funnel, and the filtrate was concentrated *in vacuo* to give 46.5 g of the 95% ethyl alcohol extract. An aliquot was dissolved in DMSO and submitted for testing. The 95% ethyl alcohol extract was found to be active and therefore was then further partitioned according to the scheme shown in Fig. 1. The testing data are shown in Table 1. All fractions were tested at the dose-response dose which was defined as the percentage of the 95% ethanol-extractable material that the fraction represented times the dose of 95% ethanol fraction used in the metabolism assay (in this case, 750 μg/ml of medium).

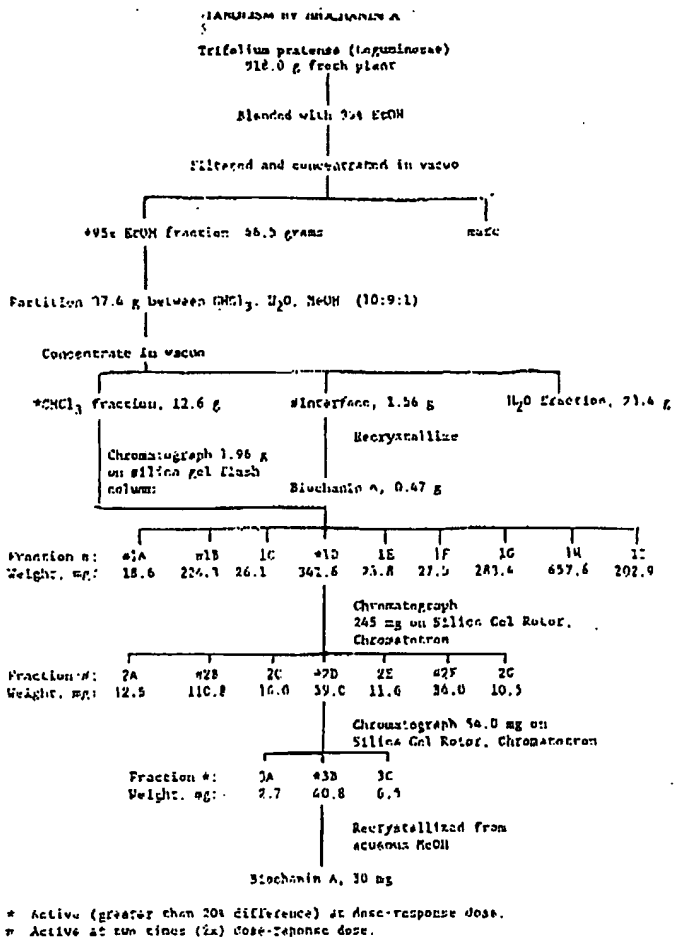
Isolation and Identification of Active Components. Aliquots from the solvent partition were submitted for testing. The active $CHCl_3$ fraction was subjected to silica gel flash column chromatography with hexane, $CHCl_3$, ethyl acetate, acetone, acetone:methyl alcohol (1:1), and finally methyl alcohol. Nine fractions were collected, and aliquots were taken and submitted for testing. The column fraction which was active at the dose-response dose (Fraction 1D) (see Fig. 1) was further chromatographed by centrifugal silica gel TLC (Chromatotron) using a $CHCl_3$/methyl alcohol solvent gradient starting with 2% methyl alcohol in $CHCl_3$. The fractions which were collected were combined according to the presence of similar spots when analyzed by silica gel TLC developed in 2% methyl alcohol in $CHCl_3$. Based upon this, the samples were combined into seven fractions which were tested for their effects on B(a)P metabolism. The most active fraction (2D) was further separated on another silica gel Chromatotron plate developed in a $CHCl_3$/methyl alcohol gradient. Based upon TLC profiles eluants were combined into three fractions. The most active fraction (3B) contained a major component. Recrystallization of this fraction from aqueous methyl alcohol gave a crystalline material, m.p. 217–218°C. A sample of authentic biochanin A was purchased from Aldrich Chemical Co., m.p. 218–219°C. A mixed m.p. showed no depression. The UV and ^{1}H NMR data were identical to literature values (12), and the MS and ^{13}C NMR data were consistent with the published structure.

Examination of the interface fraction, which was active at 2× the dose-response dose, led to the isolation of additional biochanin A, along with an analogue, formononetin (see Fig. 3). Formononetin was inactive in the metabolism bioassay. Biochanin A represented about 30% of the interface fraction.

RESULTS

The results of bioasssay-directed fractionation of the active ethyl alcohol extract of red clover are presented in Fig. 1 and Table 1. The ethyl alcohol extract was active at doses from 500 μg/ml to 1000 μg/ml; however, toxicity was detected at the highest dose (see Table 1). Further partitioning of the active ethyl alcohol extract was dose responded from 750 μg/ml. After partitioning between chloroform and water, the activity appeared in the chloroform extract. Examination of the interface which was active at twice the dose-response dose confirmed the presence of biochanin A. Chromatography of the chloroform fraction gave active column Fraction 1D. This fraction was carried through two separations on the Chromatotron to give in turn active Fractions 2D an 3B. Crystallization of Fraction 3B gave 30 mg of the active constituent, biochanin A. Fractions 1A, 1B, 2B, and 2F show activity at twice the dose-response dose and are under further investigation. The B(a)P metabolites present in the organic phase of the sample treated with red clover extract at 500 μg/ml were analyzed by HPLC, and the amount of the major primary B(a)P metabolites was determined (Fig. 2). The two major changes were a slight increase in the amount of 9-hydroxy-B(a)P and a major decrease in the amount of water-soluble metabolites in the extract-treated group. After β-glucuronidase treatment of the aqueous phase, the amount of 9-hydroxy- and 3-hydroxy-B(a)P in the red clover extract-treated group was reduced by 30% and 22%, respectively, when compared to DMSO controls. The water-soluble metabolites were also decreased by 18% in the red clover extract-treated cells. Thus the major effect of red clover extract was to inhibit the formation of B(a)P-phenol glucuronides.

The effect of the crude 95% ethyl alcohol extract on the binding of B(a)P to DNA was also examined (Table 2). At a dose of 250 μg/ml the extract inhibited B(a)P-DNA binding by 30% to 41% compared to controls in three separate experiments. Analysis of the B(a)P-DNA adducts present in enzyme-digested DNA samples by HPLC demonstrated that the extract inhibited the formation of both the *syn*- and *anti*-isomers of B(a)PDE. The *syn*-B(a)PDE-dGuo adducts decreased from 37% to 64% compared to controls, and the (+)-*anti*-B(a)PDE-dGuo adduct decreased from 48% to 75%.



* Active (greater than 20% difference) at dose-response dose.
\# Active at two times (2x) dose-response dose.

Fig. 1. Fractionation scheme for the 95% ethyl alcohol extract of fresh red clover leaves, flowers, and stems. *EtOH*, ethyl alcohol; *MeOH*, methyl alcohol.

The effect of biochanin A on the binding of B(a)P to DNA in hamster embryo cell cultures was also examined. Biochanin A caused a 54% decrease in B(a)P metabolism at 25 μg/ml. After exposure of cultures to 25 μg of biochanin A and 1 μg of [^{3}H]B(a)P per ml of medium for 24 h, biochanin A treatment reduced the amount of B(a)P bound per mg of DNA from 74.3 pmol in the control group to 35.1 pmol in the biochanin A group in one experiment and from 72.2 to 45.4 in a second experiment. Thus, biochanin A inhibited the binding of B(a)P to DNA to an extent similar to that obtained in the crude extract (Table 2).

DISCUSSION AND CONCLUSIONS

There are several bioassays which are under investigation for the detection of compounds suspected of having potential cancer chemopreventive activity. Antimutagenic activity in the form of an anti-Ames assay has been commonly used in the United States and Japan (13, 14). Mitscher *et al.* (15) used this bioassay to isolate and identify glabrene, a known isoflavene exhibiting antimutagenic activity. Nishino *et al.* (16) used the antitumor-promoting activity of glycyrrhetic acid against 7,12-dimethylbenz(a)anthracene and teleocidin as a model of cancer prevention. The decrease in formation of carcinogenic *N*-nitroso compounds produced by α-tocopherol and ascorbic acid was used as a criterion for chemoprevention by Narkus *et al.* (17) and Mervish (18). Sakiyama *et al.* (19) used the inhibition of transformation of the mouse 10T½ cell line induced by X-ray or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as a model to show the anticarcinogenic effects of lipopolysaccharides and indomethacin. The induction of aryl hydrocarbon hydroxylase activity in liver and intestinal mucosa of Sprague-Dawley rats was used by Wattenberg *et al.* (3) to isolate and identify a group of indoles from cruciferous vegetables (20, 21). Another screen by Wattenberg *et al.* (5) used the induction of glutathione *S*-transferase activity, a major detoxification enzyme system, for a number of electrophiles, including many carcinogens, in mouse liver and intestinal mucosa to isolate a group of known diterpenes from green coffee beans.

The screening procedure described in this paper measures effects on the ability of hamster embryo cell cultures to metabolize the carcinogen B(a)P. Induction or inhibition of B(a)P metabolism of treated cultures by >20% as compared with control cultures was considered to be an active test. The altered pattern of metabolism was determined by HPLC analysis of the B(a)P metabolites formed, and the effects on binding of B(a)P to DNA are determined. Confirmed active extracts are then fractionated using the bioassay as a guide. Advantages of our method are that activity data can be generated within a few days after the extract or compound is tested, and a large number

Table 1 *Activity of fractions in B(a)P metabolism assay*

The procedure used for analysis of B(a)P metabolism to water-soluble metabolites is described in "Materials and Methods."

Fraction	Dose-response dose (μg/ml medium)	% of change from control	2X dose-response dose	% of change from control
95% Ethyl alcohol	500	−39.9 ± 6.2[a, b]		
	750	−40.4 ± 6.9[b, c]		
$CHCl_3$	203	−35.6 ± 5.7[b, c]	406	−68 ± 4.6[c]
Interface	25.2	−17.7 ± 7.5[c]	50.2	−33.8[d, e]
H_2O	541	−15.4 ± 1.4[c]		
1A	1.9	17.2 ± 5.4[b]	3.9	28.8[d, e]
1B	23.3	−5.9 ± 15.2[d]	46.6	−34.6 ± 4.6[c, e]
1C	3.7	8.7 ± 5.6[b]	7.4	−8.0[d]
1D	35.5	23.5 ± 4.0[b, e]	71.0	−50.2[d, e]
1E	2.5	3.25 ± 6.8[c]	5.0	−3.5[c]
1F	2.8	8.5 ± 0.8[c]	5.6	13.6[d]
1G	29.4	12.1 ± 7.3[c]	58.8	17.8[d]
1H	68.1	5.0 ± 9.2[c]	136.2	−5.5[d]
1I	21.0	−5.9 ± 11.4[c]	42.0	−11.7[d]
2A	1.8	−14.1[d]	3.6	4.8[d]
2B	16.1	−12.9[d]	32.2	−32.7[d, e]
2C	2.3	−13.8[d]	4.6	−12.9[d]
2D	8.6	−25.3[b, d]	17.2	−19.9[d]
2E	1.7	−13.8[d]	2.4	3.0[d]
2F	4.9	−14.6[d]	9.8	−23.0[d, e]
2G	1.5	−1.8[d]	3.0	
3A	0.4	−6.8[d]	0.8	
3B	6.4	−23.7[b, d]	12.8	−23.0 ± 22.9[c, f]
3C	1.0	5.93 ± 18.6[c]	2.0	8.8[d]
Biochanin A	4.7	−12.2[d]		
	9.5	−32.1[d]		
	19.0	−47.4[d]		
	23.6	−48.8[d]		

[a] Mean ± SD of 3 experiments.
[b] Active (greater than 20% difference) at dose-response dose.
[c] Average ± range of 2 experiments.
[d] One experiment.
[e] Active at 2X dose-response dose.
[f] Mean ± SD of 4 experiments.

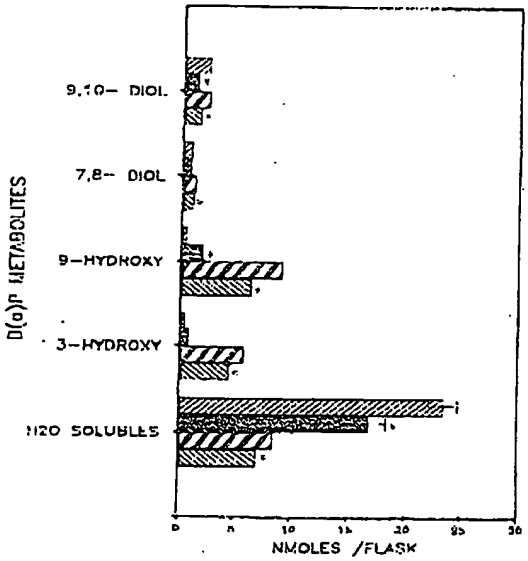


Fig. 2. The amount of B(a)P metabolites formed in hamster embryo cell cultures in the presence or absence of 500 μg/ml of red clover crude extract. The cultures were treated, and the medium samples used analyzed as described in "Materials and Methods." Medium samples were treated with β-glucuronidase prior to extraction to determine glucuronide conjugates. *Columns*, mean for 3 flasks per group; *bars*, SD. *, red clover extract-treated samples that differed significantly from the corresponding control (based upon Student's *t* test; $P < 0.01$). ▨, control; ▩, *T. pratense*, control (β-Glucuronidase); ▧, *T. pratense* (β-Glucuronidase).

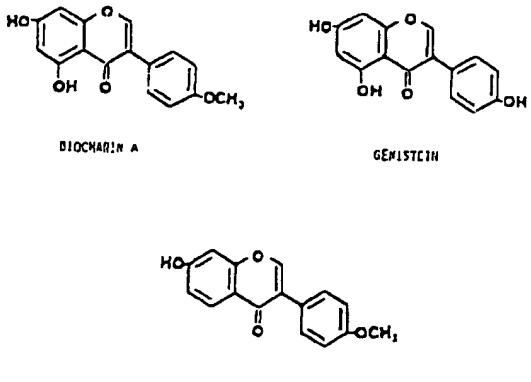


Fig. 3. Isoflavones isolated from red clover leaves and flowers (25, 26).

of different samples can be screened simultaneously. After pure active compounds are isolated and their effect on the metabolic activation of B(a)P is established, they will then be further tested using *in vivo* bioassays to determine their effect on tumor induction by various classes of carcinogens. These *in vivo* bioassays are essential for determining whether a compound acts as an anticarcinogen and against which classes of carcinogens it was active.

Thus far we have screened over 70 species and varieties of plants and vegetables comprising 27 families. One of the first plant extracts demonstrated to produce reproducible inhibition of B(a)P metabolism in the hamster cell culture assay was that prepared from red clover. Based upon inhibition of B(a)P metabolism the crude red clover extract was fractionated, and

Table 2 *B(a)P-DNA binding in hamster embryo cells treated with extracts of red clover*

Hamster embryo cell cultures were exposed to the 95% ethyl alcohol extract of red clover at a dose of 250 μg/ml of medium, and 10 min later 1.0 μg of [^{3}H] B(a)P per ml of medium was added. After 24 h, the medium was removed, and a sample was analyzed by chloroform:methanol extraction as described in "Materials and Methods." The percentage of radioactivity in the water phase is reported as the percentage of water-soluble B(a)P metabolites. The DNA was isolated from the cells, and the level of binding of B(a)P was measured. The DNA was digested to deoxyribonucleosides, and the amount of the major B(a)P-DNA adducts was determined by HPLC.

	Experiment 1		Experiment 2		Experiment 3	
	Control	Test extract	Control	Test extract	Control	Test extract
% of water-soluble B(a)P metabolites	48.4	40.5	30.8	15.4	35.6	27.5
Binding of B(a)P to DNA: total level of binding (pmol/mg DNA)	67.0	42.7	51.2	30.0	23.0	15.8
(+)-*anti*-B(a)PDE-dGuo adduct (pmol/mg)	12.7	4.9	11.1	2.7	5.5	2.9
syn-B(a)PDE-dGuo adduct (pmol/mg)	8.4	3.0	8.1	3.7	5.0	3.3

a pure active compound, biochanin A, was isolated which produced an inhibition of B(a)P metabolism of 30 to 50% at 9.5 to 23.6 μg/ml compared to DMSO controls. Exposure of hamster embryo cell cultures to biochanin A at a dose of 25 μg/ml of medium resulted in a 37 to 50% inhibition in the binding of B(a)P to DNA. This compound appears to be one of the major components responsible for the inhibition of B(a)P-DNA interactions by the red clover extract. The strong correlation between the binding of aromatic hydrocarbons to DNA and their carcinogenic activity suggests that biochanin A is a good candidate for further testing to measure inhibition of tumor induction by hydrocarbons in animals.

Several flavonoids have been shown to possess anticarcinogenic activity (3). 7,8-Benzoflavone, a synthetic flavonoid, is an inhibitor of microsomal mixed-function oxidases and inhibits the metabolism, binding to DNA, and tumorigenesis of 7,12-dimethylbenz(*a*)anthracene in mouse skin (22). This same flavonoid also inhibits the metabolism of B(a)P in rat hepatic microsomes that have been induced with 3-methylcholanthrene (23). Huang *et al.* (24) examined 28 flavonoids for their effect on mutagenicity of anti-B(a)PDE in *Salmonella* and found that 8 had significant antimutagenic activity. Interestingly one of the flavonoids found to be inactive (50% inhibitory dose > 100) was genistein, an isoflavone related to biochanin A (see Fig. 3) and a minor constituent of red clover (25, 26). Since the compound tested [B(a)PDE] was an ultimate mutagenic metabolites of B(a)P, that assay would not be expected to detect compounds that alter metabolic activation of B(a)P. Thus, various types of short-term assays may be anticipated to detect anticarcinogens that work by different mechanisms. In view of the requirement of the majority of classes of chemical carcinogens for metabolic activation and the ability of the metabolism assay to measure changes in enzymes both involved in activation as well as detoxification, the hamster cell assay should be capable of detecting modifiers of carcinogen metabolism that act by a number of mechanisms. The results demonstrate that the effects of test compounds on B(a)P metabolism and DNA binding in hamster embryo cell cultures can be used to screen and isolate pure compounds with potential anticarcinogenic activity from plants and other natural products.

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SHORT PAPER

OESTROGENIC ACTIVITY OF SOYA-BEAN PRODUCTS

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Abstract—Normal rat cake containing soya meal was found to be oestrogenic. Sixteen samples of soya meal were examined in the mouse uterine weight bioassay and all were found to have oestrogenic activity. Ethyl-acetate extracts of the meals also had oestrogenic activity. Genistein and daidzein were present in the extracts.

Introduction

It has previously been reported from this laboratory (Drane, Patterson, Roberts & Saba, 1975) that rat cake used as a control feed in routine mouse bioassays for oestrogens had developed significant uterotrophic activity over a period of a few months. We have recently encountered another oestrogenic control feed, higher basal uterine weights than expected being found in mice fed this ration. Investigation of the components of the feed showed that the oestrogenic activity was due to soya meal, which made up 10% of the rat cubes.

Little attention seems to have been paid to soya meal as a possible source of oestrogenicity although daidzein and genistein were isolated from soya beans nearly 50 yr ago (Walz, 1931). The oestrogenic activity of these and other isoflavones is well documented (Bickoff, Livingston, Hendrickson & Booth, 1962; Carter, Smart & Matrone, 1953; Cheng, Story, Yoder, Hale & Burroughs, 1953). A new isoflavone, glycitein, has been isolated from soya beans (Naim, Gestetner, Kirson, Burk & Bondi, 1973) and recently coumestrol was also shown to be present at levels ranging from 0·05–30 μg/g (Lockhart, Jones & Finney, 1978). The present report provides bioassay data showing that oestrogenic activity was present in all sixteen samples of soya meal examined.

Experimental

Materials. Sample 1 was the extracted soya-bean meal that had been used in the manufacture of the control feed (Porton Rat Diet) associated with the original problem. Samples 2–14 were soya-bean meals of various origins destined for the manufacture of farm-animal feeds. Sample 11 was a pelleted form of feed. Samples 15 and 16 were soya-bean products intended for human consumption. Semi-synthetic (SS) feed supplied by RHM Labsure Ltd. was used as a soya-free control.

Three extracts were prepared. For the first, 90 g soya-bean meal was exhaustively extracted with ethyl acetate in a Soxhlet apparatus. The solvent was evaporated to dryness and the residue was re-dissolved in a convenient volume of ethanol–ethyl acetate (1:1, v/v). A second, 70%-ethanol extract was prepared as described for the extraction of oestrogens from white clover (Saba, Drane, Hebert & Holdsworth, 1974). A third extract in aqueous acetonitrile was also prepared (Drane *et al.* 1975).

Oestrogen bioassay. Eighteen-day-old MF1 weanling female mice weighing 7–9 g were supplied by OLAC 1976 Ltd., Bicester, Oxon. They were housed in groups of six to a cage and each group was given 40 g of feed over a period of 3·5 days. On the following day the mice were killed and the uteri were dissected out, blotted on filter paper and weighed. Each assay included a control group given only the SS feed and three or four groups given SS feed containing known amounts of diethylstilboestrol (DES). The test soya meal samples were fed alone, or mixed with SS diet, or as an extract mixed into SS diet and air dried.

Mycology and mycotoxin screening. Samples of soya-bean meal were screened for possible mycotoxin contamination by the method described by Roberts & Patterson (1975) as modified by Patterson & Roberts (1979). The mycological examination of six samples was carried out by the methods described by Shreeve, Patterson & Roberts (1975).

Thin-layer chromatography (TLC). Biologically-active ethyl-acetate extracts were examined for phyto-oestrogens by TLC using Polygram Sil G/UV_{254} sheets and methanol–chloroform (7:93, v/v) as the developing solvent. Genistein, daidzein and formononetin (minimum detectable levels 10 μg/g) and coumestrol (minimum detectable level 1 μg/g) were run as reference compounds. The developed chromatogram was examined under long- (360 nm) and shortwave (250 nm) ultra-violet light for fluorescing and absorbing spots both before and after exposure to ammonia vapour. These active extracts were also analysed for zearalenone using the mycotoxin method cited above (analytical limit 20 μg/kg).

Results and Discussion

No mould growth was evident in any of the six soya meals sampled and Fusarium species were not

isolated in mycological cultures of the meals. Neither zearalenone nor any other mycotoxin was detected.

The mouse uterine weight bioassay data are summarized in Table 1. Samples 2 and 3 were oestrogenic when fed as whole meal but their extracts were not tested. Nine other samples were active when fed as whole meal and also when fed in the form of ethyl-acetate extracts, while five further samples were active only when fed as ethyl-acetate extracts. Thus all sixteen samples showed biological activity. No oestrogenic activity was found in extracts in 70% aqueous ethanol, which is routinely used to extract substances with oestrogenic activity from red and white clover (Bickoff, Loper, Hanson, Graham, Witt & Spencer, 1967; Saba *et al.* 1974). Neither was it found in extracts in acetonitrile, which has previously been used to isolate an active fraction 6bII from oestrogenic rat cake (Drane *et al.* 1975). However, all of the ethyl-acetate extracts were oestrogenically active, although when appropriate comparisons were made, it was found that the recovery of the source of the activity present in the original samples of soya meal was poor. Hydrolysed ethyl-acetate extracts examined by TLC were found to contain genistein and daidzein and preliminary experiments suggested that the former isoflavone contributed most of the oestrogenic activity. No other reference oestrogen was detected.

Various reproductive disturbances in animals have been traced to the ingestion of oestrogenic feeds. Cattle became infertile whilst grazing lucerne containing high concentrations of coumestrol (Adler & Trainin, 1967), hyperoestrogenism was reported in pigs fed diets containing 0·1–6·8 μg zearalenone/g (Mirocha, Pathre & Christensen, 1977), the conception rate was lowered in sheep fed 8–16 μg DES/day and conception was prevented altogether in sheep given 32 μg DES/day (Morley, Bennett & Axelsen, 1963). The present results suggest that comparable levels of oestrogenic activity might be provided by diets containing soya products; in those whole soya meals in which quantifiable amounts of oestrogenic activity were present, levels equivalent to 8–37 ng DES/g soya were detected. On the basis of our own estimate that the potency of the mycotoxin zearalenone in the mouse bioassay is $8{\cdot}5 \times 10^{-4}$ that of DES, the observed oestrogenic activity of these soya meals was equivalent to 9·4–43·3 μg zearalenone/g soya.

There is little published information on the oestro-

Table 1. *Oestrogenic activity of whole soya meal and of ethyl-acetate extracts of whole soya meal in the mouse uterine weight assay*

	Results for mice fed whole soya meal				Results for mice fed ethyl-acetate extracts of soya meal			
Sample no.	Maximum total dose† (g whole soya meal/mouse)	Uterine wt (geometric mean; mg)	DES equivalent (ng/g soya)	Calculated zearalenone equivalent‡ (μg/g soya)	Maximum total dose† (g soya meal extracted/ mouse)	Uterine wt (geometric mean; mg)	DES equivalent (ng/g)	Calculated zearalenone equivalent‡ (μg/g soya)
1	6	10·4*	<10§		NT			
	6	16·5**	8	9·4	15	19·4***	5	5·9
2	6	11·7	<10		NT			
	6	13·9*	17	19·9	NT			
3	6	16·0***	10–20		NT			
	6	17·4***	17	19·9	NT			
	6	19·0***	10	11·7	NT			
4	6	11·3***	<20		NT			
	6	13·6***	10	11·7	20	18·6***	3·5	4·1
5	3	12·2**	<10		15	16·6***	4	4·7
6	3	8·5	0·0		15	19·0***	5	5·9
7	3	11·0*	<10		14	33·5***	8	9·4
8	2·5	6·3	0·0		12	29·1***	7	8·2
	5	10·5	<10		NT			
9	2·5	7·7	0·0		14	21·3***	4	4·7
	5	9·0	<10		NT			
10	2·5	10·1**	<10		12	24·0***	5	5·9
11	3	7·8	0·0		12	15·5***	3	3·5
12	2·5	9·9	<10		12	22·8***	5	5·9
13	3	31·5***	37	43·3	15	56·5***	12	14·0
14	3	13·6***	12	14·0	15	32·7***	7	8·2
15	5	34·0***	24	28·1	15	36·3***	8·7	10·2
16	4	15·5***	16	18·7	15	25·3***	7	8·2

NT = Not tested

†The mice ate poorly, and therefore the dose is only approximate.

‡By the mouse uterine weight assay the zearalenone equivalent per unit wt of DES = 1170 (850–1600).

§Oestrogenic activity present at levels equivalent to <10 ng DES/g soya could not be quantified.

The values marked with asterisks differ significantly from those of the controls that were given soya-free semi-synthetic feed (*$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$). Sample 1 was a soya-bean meal used in the manufacture of rat feed. Samples 2–14 were soya-bean meals used for the manufacture of farm-animal feeds. Samples 15 and 16 were soya-bean products intended for human consumption.

genic activity of foodstuffs for human consumption (Schoental, 1977) and this report helps to remedy the situation. Since soya meal is an important source of protein for animal feeds and is now increasingly used in human food, we feel that this apparently constant source of oestrogenic activity should not be overlooked, even though it is at a low level. However, species differ greatly in their susceptibility to the effects of oestrogens and caution must therefore be exercised when attempting to extrapolate data from species to species or from the biological effects of one oestrogenic substance to another.

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DIETARY PHYTOESTROGENS AND CANCER: *IN VITRO* AND *IN VIVO* STUDIES

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Summary—Thirty postmenopausal women (11 omnivores, 10 vegetarians and 9 apparently healthy women with surgically removed breast cancer) were investigated with regard to the association of their urinary excretion of estrogens, lignans and isoflavonoids (all diphenols) with plasma sex hormone binding globulin (SHBG). A statistically significant positive correlation between urinary total diphenol excretion and plasma SHBG was found which remained statistically significant after elimination of the confounding effect of body mass determined by body mass index (BMI). Furthermore we found a statistically significant negative correlation between plasma SHBG and urinary excretion of 16α-hydroxyestrone and estriol which also remained significant after eliminating the effect of BMI. Furthermore we observed that enterolactone (Enl) stimulates the synthesis of SHBG by HepG2 liver cancer cells in culture acting synergistically with estradiol and at physiological concentrations. Enl was rapidly conjugated by the liver cells, mainly to its monosulfate. Several lignans and the isoflavonoids daidzein and equol were found to compete with estradiol for binding to the rat uterine type II estrogen binding site (the s.c. bioflavonoid receptor). It is suggested that lignans and isoflavonoids may affect uptake and metabolism of sex hormones by participating in the regulation of plasma SHBG levels and in this way influence their biological activity and that they may inhibit cancer cell growth like some flavonoids by competing with estradiol for the type II estrogen binding sites.

INTRODUCTION

Weakly estrogenic diphenolic compounds, belonging to the classes of lignans (Ligs) and isoflavonoids (Ifls), are excreted in large amounts in human (and animal) urine. Subjects consuming whole-grain products, seeds, fruits and berries (contains mammalian lignan precursors) and soy products (contains isoflavonoids, and lignan precursors) [1–6] have high excretion of these compounds. Up to now about 15 structurally different compounds were isolated and identified by combined gas chromatography–mass spectrometry (GC/MS) [structures and literature in 4, 6, 7]. Intestinal bacteria play an important role in the transformation of the plant precursors [2, 7, 8].

Lignan excretion in women is usually high in areas with low risk for breast cancer (BC) like North Karelia in Finland [4], and in vegetarians [4, 5, 9, 10] and low in women living in high-risk areas like Boston, U.S.A. [4, 5, 10]. In old women with BC in Boston the excretion was very low [10] and it was also relatively low in Finnish young women with BC [9]. On the other hand we also found low excretion of Ligs in Japanese women consuming traditional Japanese diet and having low BC risk. However these subjects excreted very high amounts of Ifls, particularly genistein (Gen) and daidzein (Daid) [11]. There is already evidence suggesting that both Ligs and Ifls are protective with regard to BC [12–17] and that Ifls may be protective with regard to prostate cancer (PC) [16, 18].

In the present study we continue to explore the link between the Ligs and Ifls, and hormone-dependent cancer and the possible mechanisms by which the cancer-protective effect of these compounds is exerted. The results obtained strongly suggest that these compounds have cancer-protective properties.

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MATERIALS AND METHODS

Subjects and their diet

In this connection only some preliminary *in vivo* results with regard to plasma sex hormone binding globulin (SHBG), urinary estrogens, Ligs and Ifls for the last part of the "Finlandia" study involving postmenopausal women will be described. The groups studied were 11 omnivorous and 10 vegetarian women and 9 apparently healthy women with breast cancer (BC) treated with surgical removal of the breast (Stage I and II). Simultaneously with the collection of urine and blood samples, very careful dietary records during 5 days were obtained, once in winter and once in the summer time. The dietary differences were surprisingly small. Preliminary calculations showed that the vegetarians had higher intake of total fiber (22.7 g/day, geometric means) than the omnivores (16.6 g/day) and the BC patients (16.0 g/day) but this was statistically significant only when compared with the BC group ($P < 0.04$). No significant differences in dietary intake between the omnivorous and BC groups could be observed. Cholesterol intake was significantly lower in the vegetarians (omnivores vs vegetarians $P < 0.02$; BC vs vegetarians $P < 0.002$). Furthermore we found a statistically significantly higher intake of vegetable fiber in the vegetarians compared to the BC group (vegetarians 4.5 g/day and BC 2.3 g/day, $P < 0.03$). Complete dietary data will be published elsewhere.

Collection of blood and urine samples

The women collected 72-h urine samples and three different blood samples were drawn between 8 and 9 a.m. into heparinized tubes on the same consecutive days. The plasma was pooled and the samples were stored with 0.1% ascorbic acid and 0.1% sodium azide at −20°C until analyzed. In the present study the mean values for one winter and one summer collection period were used (6 plasma and 2 × 72-h urine samples for each subject).

Reference standards and deuterium-labelled compounds

The Ligs Enl, End, matairesinol (Mat), and the Ifls, Daid, Equol and *O*-desmethyl-angolensin (*O*-Dma) were synthesized and the preparation of the deuterium-labelled standards was carried out as described previously [lit. in 19]. The isoflavonoid Gen was a generous gift from Professor K. Kallela.

Cell cultures

Prior to the growth experiments the cells (HepG2 liver cancer and MCF-7 breast cancer cells, American Type Culture Collection, Rockville, Md, U.S.A.) were maintained in Dulbecco's modified Eagle's medium (DMEM) without phenol red supplemented with 10 mM of L-glutamine, 100 IU/ml of penicillin, 100 IU/ml of streptomycin, 1% (v/v) NEAA, and 15 mM Hepes (Boehringer Mannheim, Fed. Rep. Germany) with 10% fetal calf serum (FCS). Before the experiment the cells were detached by removing the medium and washing with ice-cold Ca- and Mg-free phosphate buffered saline (PBS) (Orion Diagnostics, Espoo, Finland) and trypsinization (trypsin 0.05%, EDTA 0.02%). 100,000 to 400,000 cells, depending on the size of the plastic petri dishes, were plated in the same medium but now with 5% FCS for two days. The medium was removed, cells washed with PBS, and fresh medium with 5% twice DCC-treated FCS added and incubated for a further 3 days. The preparation of the DCC-treated FCS was carried out as described [15].

After the final washing of the cells twice with ice-cold PBS and added fresh medium with 5% DCC-treated FCS, the cells received effectors in ethanol solution to a final concentration of not more than 0.1% ethanol. The cells were maintained at 37°C in a 100% humid atmosphere of 92% air and 8% carbon dioxide as a monolayer culture in Falcon's plastic petri dishes (9 cm dia.) or in dishes with six 2.5 cm wells. The effectors were added once per day and the medium changed every fifth day. Duration of experiments was 8–10 days. Cells were counted both manually in a Bürker chamber and using the Coulter counter industrial cell counter (Coulter Electronics Ltd, Luton, Beds., England). DNA was measured by fluorometry with a slight modification of the original procedure [20] using the Transcon 102 FN fluoronephelometer (Elomit Oy, Helsinki, Finland) and the results were expressed in pmol/mg DNA.

Cell cultures in metabolite studies and determination of enterolactone conjugates

In the metabolite studies with HepG2 cells, the cells, after the initial treatment described above, were first grown for four days as described and every morning Enl was added to a final concentration of 1 μM. After four days the medium was removed and the cells washed with

ice-cold PBS and fresh medium added. Thereafter the procedure was continued for another 4 days and 24 h after the last addition of Enl extraction was carried out as described [15]. The fractionation of Enl conjugates and their determination was carried out as previously described [21] with slight modifications [15]. To the final fractions 211.2 ng $^2H_6$-labelled Enl was added in 50 μl of ethanol, the solvent evaporated to dryness and the samples silylated. After trimethylsilyl ether derivative formation the solvent was evaporated to dryness, the residue was dissolved in a suitable amount of n-hexane and the quantitation carried out by GC/MS in the selected ion monitoring (SIM) mode as described [22].

In studies on the time course of Enl conjugation, 1 million HepG2 cells were plated and Enl added to a final concentration of 2 μM. Samples were taken at various time intervals up to 74 h. Medium was extracted with ethyl ether and the conjugates hydrolyzed with *Helix pomatia* extract as described [22] and the liberated aglycone extracted with ether and assayed by GC/MS.

Determination of SHBG in the medium

SHBG assays in the medium were carried out with a highly sensitive time-resolved fluoroimmunoassay (TR-FIA) using reagents provided by Farmos Ltd (Turku, Finland).

Studies of the binding of diphenols to the nuclear type II estrogen binding site

Adult ovariectomized rats were implanted with 20 μg of estradiol (E2) and 96 h after treatment uterine nuclear fractions were prepared. The various lignans and isoflavonoids were dissolved in Tris–EDTA buffer containing 20% ethanol and their ability to inhibit the binding of [^{3}H]estradiol (40 nM) to nuclear type II sites were assessed [23, 24].

Assays of estrogens, lignans and isoflavonoids in urine and SHBG in plasma

Ligs and Ifls were determined in urine by an isotope dilution gas chromatographic–mass spectrometric method recently described [19] combining the method with the estrogen profile method also described previously [22]. This allows simultaneous assay of 20 compounds. In the present study Mat and Gen were not assayed because the method did not include these two compounds at the time of analysis. Thus we determined 13 estrogens and the Ligs Enl and End, and the Ifls Daid, Equol, and *O*-Dma. SHBG in plasma was determined by the RIA kit provided by Farmos Ltd.

Statistical methods

The mean values presented are geometric means. In the statistical analyses the mean values for the winter and summer collection periods were used and when necessary logarithmic transformation was made because of skewness of the distribution of the results. The degree of univariate associations between two variables was estimated as Pearsons correlation coefficients (r). Partial correlations were calculated to eliminate the effect of body mass index (BMI) on the results. Correlation coefficients and partial correlation coefficients were calculated using the StatView II programme for Macintosh II (Abacus Concepts, Inc. Berkely, CA, U.S.A.).

RESULTS

SHBG, diphenols and estrogen 16α-hydroxylation

In the three groups of postmenopausal women the plasma SHBG values were statistically significantly highest in the vegetarians (70.3 nmol/l) ($P < 0.0002$) compared to the omnivores (31.1 nmol/l) and BC patients (34.8 nmol/l). The vegetarians had significantly higher urinary excretion of Enl, total Ligs, total Ifls, and total diphenols ($P < 0.05$–$P < 0.007$) compared to the two other groups (details to be published elsewhere).

We found a statistically significant positive correlation between urinary excretion of *O*-Dma, Enl, total lignans and total diphenols and plasma SHBG. However, this correlation was partially dependent on the fact that the vegetarians had significantly lower body mass (BMI = omnivores 26.1, vegetarians 22.4, BC 26.2). BMI showed a negative correlation with SHBG ($r = -0.580$; $P < 0.001$) in these subjects. After elimination of the confounding effect of BMI we still found a statistically significant positive association between the urinary excretion of *O*-Dma ($r = 0.421$), Enl ($r = 0.391$), total Ligs ($r = 0.382$) and total diphenols ($r = 0.400$) and plasma SHBG ($P < 0.05$ for all).

When studying the association between plasma SHBG and the excretion of individual urinary estrogens we found that there was no association between SHBG and urinary catecholestrogens. However, we found statistically

significant negative correlations between SHBG and urinary 16α-hydroxyestrone ($r = -0.448$; $P < 0.05$) and estriol ($r = -0.572$; $P < 0.001$). Partial correlation coefficients eliminating the linear effect of BMI on the results showed that these significant associations remained but were weaker ($r = -0.390$ and $r = -0.409$ for 16α-hydroxyestrone and estriol, respectively, both $P < 0.05$).

Stimulation of SHBG synthesis by enterolactone in HepG2 liver cell cultures

Concentrations of Enl between 0.5 and 10 μM stimulated SHBG synthesis by HepG2 human liver cancer cells in culture (Fig. 1). The maximal effect was found with 5 μM concentration and a toxic effect could be observed with concentrations above 10 μM (Fig. 1). 200 nM concentration of estradiol (E2) was needed to obtain a similar stimulation of SHBG synthesis as 2 μM of Enl. When E2 (200 nM) and Enl (5 or 10 μM) were combined they had additive effects on the synthesis (Fig. 1).

Metabolism of enterolactone by HepG2 liver cells

The conjugation of Enl by HepG2 cells was very rapid and within 10 h more than 95% was conjugated (Fig. 2). The relative concentrations of the different conjugates of Enl identified in the medium are shown in Table 1. The main conjugate was the monosulfate (EnlS) amounting to about 78% of the total.

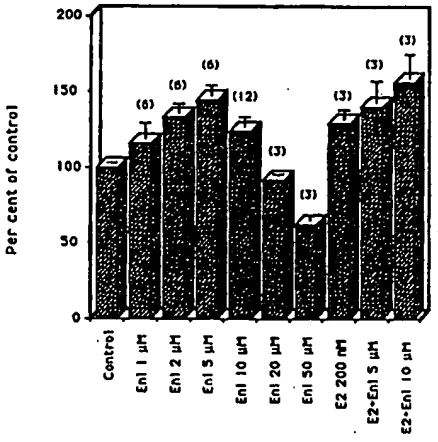

Fig. 1. Enterolactone stimulation of sex hormone binding globulin (SHBG) synthesis by HepG2 cells in culture in the absence and presence of estradiol. Number of experiments indicated on the top of the bars.

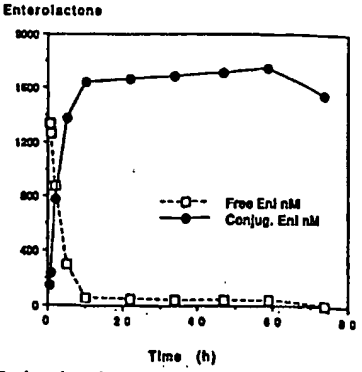

Fig. 2. Conjugation of enterolactone (Enl) (2 μM) added to cultures of HepG2 cells.

Binding of diphenols ot the nuclear type II binding site (bioflavonoid receptor)

Figure 3 (top) shows the binding of the two main mammalian lignans Enl and End to the nuclear estrogen type II binding site. In addition the binding of two plant lignans, matairesinol, which is the precursor of Enl [2, 7] and of isolariciresinol is shown. In the lower part of the figure it can be seen that daidzein and equol show significant binding but their precursor formononetin does not bind to the bioflavonoid receptor.

DISCUSSION

It has been proposed that a low rate of 2-hydroxylation and high rate of 16α-hydroxylation leads to a greater risk for BC and endometrial cancer. BC patients, women with genetic predisposition for BC and mouse strains with high incidence of BC have been shown to have high 16α-hydroxylation of estrogens [25–27]. Furthermore a parallel increase in *ras* proto-oncogene expression and of estradiol-16α-hydroxylation in human mammary terminal duct-lobular units by a carcinogen has been found [28].

Table 1. Distribution of conjugated metabolites of enterolactone in the culture medium 24 h after the last addition of enterolactone (1 μM) to the medium of HepG2 liver cancer cells in culture[a]

Fraction	%	Fraction	%
Unconjugated	0.12	Monosulfates	77.6
Monoglucuronides	6.29	Disulfates	5.33
Diglucuronides	6.82	Sulfoglucuronides	1.80
In other fractions	2.04		
Total	100.0		

[a]Means of two experiments.

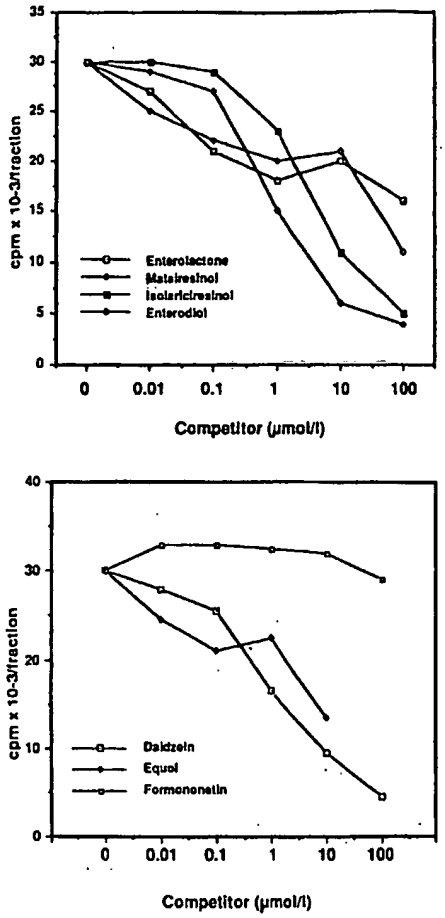

Fig. 3. Competition with [^{3}H]estradiol of lignans (top) and isoflavonoids with regard to rat uterine estrogen nuclear type II binding sites (bioflavonoid receptor).

Several earlier studies by others as well as our own seem to speak against the hypothesis that increased estrogen 16α-hydroxylation is a risk factor for BC, because all low-risk groups, compared to high-risk groups, have relatively more urinary 16α-hydroxylated estrogens, particularly if also the fecal estrogens are included. These observations have recently been discussed [16]. In addition we could previously not observe any increase in 16α-hydroxylated estrogen metabolites in urine of Finnish premenopausal women with BC compared both to omnivorous and vegetarian controls [29].

However, in the present study the old Finnish women showed statistically significant negative correlation between plasma SHBG and urinary 16α-hydroxyestrone and estriol in the whole material ($n = 30$) with the highest values of 16α-hydroxylated estrogens and lowest SHBG values in the women with BC and the omnivorous women. In the same material there was a significant positive correlation between urinary total diphenol excretion and plasma SHBG. All these associations remained statistically different despite elimination of the confounding effect of BMI on the results, although the associations were weaker. It has been shown in many studies that a low SHBG level means a higher metabolic clearance rate and uptake of sex hormones in many tissues including the liver, the principal site of estrogen 16α-hydroxylation. Postmenopausal women with BC have frequently central obesity and low SHBG levels [30–32] and we therefore suggest that some of the *in vivo* results obtained in BC patients showing increased estrogen 16α-hydroxylation may have been at least partly due to a low SHBG in the studied subjects. To our knowledge SHBG was never measured in these studies. In fact it is possible that increased cellular membrane permeability for nonpolar estrogens caused by different mechanisms may also in other tissues lead to increased 16α-hydroxylation.

Estradiol has been found to stimulate the *in vitro* synthesis of SHBG by HepG2 cells in culture, but the concentrations needed for significant increase in production are much higher (0.5–5 μM) than those occurring physiologically [33]. Our experiments show that only 10 times more Enl is needed to show the same stimulation of SHBG formation as that observed for E2. By relating SHBG synthesis to cell number and DNA it could be observed that this was not due to increased cell proliferation but to a true increase in synthesis. This was also confirmed by measuring intracellular SHBG after sonication of the cells.

The question arises whether the concentrations of Enl in the organism, particularly in the portal vein blood, are sufficiently high to have a stimulatory effect on SHBG synthesis. It is well known that estrogens administered orally, compared to parenteral administration, are much more effective in stimulating SHBG synthesis [34]. Enl enters the liver via the portal vein probably in much higher concentrations than those occurring in peripheral plasma. We know very little about the levels of Enl in plasma. Total Enl (free + conjugated) values in 4 women ranged between 0.7 and 5.3 nM [35].

Our own unpublished preliminary observations suggest that the concentrations are much higher in plasma of Finnish women and in vegetarians. We observed total Enl values between 15 and 70 nM and between 20 and over 1000 nM in omnivores and vegetarians, respectively. About 5–30% of the total occurs in the form of unconjugated Enl or in the sulfate form. As found for estrone sulfate, we believe that the sulfates of the lignans can be hydrolyzed at the cell membranes and have biological activity because of the abundance of intracellular sulfatases in the organism. Thus it is very likely that Enl in the free + sulfate form occurs in concentrations at least 10 times higher than those of unconjugated + sulfate-conjugated E2, particularly in the portal vein blood. This makes it very likely that these compounds may be involved in regulation of SHBG levels in plasma in agreement with the positive correlations observed in this and previous studies [5, 9] between excretion of lignans and isoflavonoids in urine and plasma SHBG.

Compared with MCF-7 BC cells [15], the HepG2 cells conjugate Enl as rapidly (Fig. 1 and Table 1), but less monosulfates and higher amounts of glucuronides and disulfates are formed. The monosulfates represented 78% of the total compared to 91% for the MCF-7 cells. Thus our preliminary results in plasma showing considerable amounts of sulfate-conjugated Enl in circulation are in good agreement with the *in vitro* metabolic results obtained with HepG2 cells.

Our results show (Fig. 3) that diphenolic lignans and isoflavonoids compete with E2 for the rat uterine nuclear estrogen type II binding site. These sites seem to constitute a component of the genome which regulates estrogen-stimulated uterine growth [23, 24]. Originally it was observed that some flavonoids like luteolin, quercetin and pelargonin inhibit E2 binding to this receptor and in this way uterine cell growth. They also inhibited growth of MCF-7 cells in culture, and *in vivo* E2 stimulation of immature rat uterus [36]. The structures of these flavonoids are very similar to those of the isoflavonoids. Luteolin, quercetin and perlargonin have to our knowledge not been identified in the human organism. However, Daid, Eq, Enl and End were all found in plasma, saliva and urine of human subjects and Enl, End and Eq in prostatic fluid [37, and unpublished, see above]. Now also Gen, Mat and *O*-Dma have been detected in plasma in our laboratory.

It was suggested that the isoflavonoids and flavonoids may all act synergistically inhibiting cell growth in malignant cells via the type II binding site [16] also called the bioflavonoid receptor [36, 38] or by inhibiting specifically the tyrosine protein kinase [16] the enzyme mediating the activity of many growth factors in the cell.

It is concluded that lignans and isoflavonoids may influence sex hormone metabolism and cancer by influencing plasma SHBG levels resulting in lower uptake and less biological activity of these steroids and by inhibiting growth and proliferation [13–15, 18] of hormone-dependent cancer cells.

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D10

Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet[1-4]

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ABSTRACT Epidemiologic studies revealed low mortality in hormone-dependent cancer in Japanese women and men consuming a traditional diet. We previously found that certain diphenolic food components, lignans and isoflavonoids, which are converted to biologically active hormone-like substances by intestinal microflora, may be cancer-protective agents. Therefore, we studied urinary excretion of these compounds (enterolactone, enterodiol, daidzein, equol, and *O*-desmethylangolensin) in 10 women and 9 men in a rural village south of Kyoto, Japan. The subjects consumed a typical low-fat diet with much rice and soy products, fish, and vegetables. An isotope-dilution gas chromatographic-mass spectrometric method was used for the assays. The urinary excretion of lignans was low but that of the isoflavonoids was very high. The excretion of isoflavonoids correlated with soybean-product intake. The low mortality in breast and prostate cancer of Japanese women and men, respectively, may be due to the high intake of soybean products. *Am J Clin Nutr* 1991;54:1093-1100.

KEY WORDS Japanese, diet, urine, lignans, isoflavonoids, enterolactone, enterodiol, daidzein, equol, genistein, *O*-desmethylangolensin, soybean, gas chromatography, mass spectrometry, sex-hormone-binding globulin

Introduction

Mammalian lignans and isoflavonoid phytoestrogens, occurring in all studied animal and human biological fluids and in feces, are diphenolic compounds with molecular weights similar to those of steroid estrogens (1-3). Precursors in plants seem to occur as glycosides (4, 5), and the mammalian compounds are produced from plant lignans and isoflavonoids by intestinal microflora (6-8). Most of the original plant aglycones, such as formononetin, matairesinol, and secoisolariciresinol, occur only in very low concentrations in urine (9, 10). All compounds investigated so far are weakly estrogenic but have shown many other biological activities, producing antiestrogenic (1-3); antiviral (11, 12); and antiproliferative, cytotoxic, and growth-inhibiting effects (3, 13-15). Studies indicate that they most likely stimulate the production of sex-hormone-binding globulin (SHBG) in the liver (2, 14-18) and may in this way significantly influence biological activity of the sex hormones. The higher SHBG values seen in vegetarians (2, 17-19) are probably due to the effect of these diphenolic compounds on liver synthesis of the protein (14). Studies in both young and old women with breast cancer and in various dietary groups indicate that urinary excretion of these compounds is highest in vegetarians and lower in omnivores and breast-cancer patients (2, 18, 20). It was shown that their urinary excretion correlates with the intake of fiber-rich food (2, 17, 18).

Japanese women and women of Japanese origin in Hawaii consuming a diet similar to the original traditional Japanese diet have low breast-cancer incidence and mortality (21-24). Similarly, Japanese men have low mortality with prostate cancer, although autopsy studies have found that the incidence of prostate cancer in Japanese and Western men are similar (25-27). These cancers are sex-hormone dependent and could potentially be influenced both by alterations of sex-hormone metabolism caused by lignans and isoflavonoids or by a direct effect of these compounds on their growth. Because of the associations between diet and these diseases, we decided to study the urinary excretion of lignans and isoflavonoid phytoestrogens in groups of Japanese men and women consuming a traditional diet. A preliminary report was published as an abstract (28).

Subjects and methods

Participants

The subjects participating in this investigation were apparently healthy and were recruited in a small rural village south of Kyoto,

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[2] Preliminary report published as an abstract.

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Japan. Two of the women were found to have hypertension (blood pressure 146/96 and 180/100, respectively). Most of the participants were farmers cultivating tea and rice. Originally 10 men and 10 women volunteered for the study, but 1 man was dropped because his urine volume was not known. Their main work was in agriculture and they consumed mainly their own products. The ages of the men and women were 50.4 ± 18.0 and 46.8 ± 11.5 y, respectively. Height, weight, and body mass index [BMI, in weight (kg)/height (m)^2] were, respectively, 160.8 ± 7.8 cm, 58.6 ± 5.8 kg, and 22.7 ± 2.3 for men and 153.1 ± 6.5 cm, 52.9 ± 7.2 kg, and 22.6 ± 3.5 for women. All subjects were within 15% of normal weight.

Collection of samples

Urine was collected for 48 h in plastic bottles containing 2 g ascorbic acid. The bottle was kept in a cool place during collection. The urine was mixed and measured and a sample was frozen as soon as possible and transported to Finland in dry ice for analysis.

Dietary data

The study was carried out in October 1985. Before the survey a nutritionist explained how to weigh the food components and how to write down the results on a form. Most of the food was weighed. Some food, such as bread and milk, was recorded as a piece of bread or cup of milk and the nutritionist estimated the weight of these food items afterwards. Food intake was recorded for 3 d and the nutritionist followed all subjects every day during the survey period. Calculation of the food data was made by an experienced nutritionist using the *Standard Tables of Food Composition in Japan* (29); for fiber calculations the *Food Composition Tables of Dietary Fibers, Minerals, Cholesterol, Fatty Acids* was used (30). The amount of soy sauce in the diet was calculated from the total sodium chloride content of the urine. According to earlier studies Japanese obtain 25.8% of their sodium chloride from soy sauce (31). Soy sauce contains 15% NaCl. The consumption of soy sauce is estimated by using the following formula:

$$\text{Soy sauce} = (\text{amount of NaCl in urine}) \times 0.258/0.15$$

This is the traditional way to estimate soy sauce consumption in Japanese subjects because they do not add any other salt to their food. It is an estimate and not an exact figure and the values were not included in the correlation analyses.

Analytical method

The trivial and systematic names of the compounds measured and discussed are as follows [structures were shown previously (3)]: enterolactone (Enl), *trans*-2,3-bis[(3-hydroxyphenyl)methyl]-γ-butyrolactone; enterodiol (End), 2,3-*bis*[(3-hydroxyphenyl)-methyl]-butane-1,4-diol; daidzein (Da), 4',7-dihydroxyisoflavone; equol (Eq), 4',7-dihydroxyisoflavan; *O*-desmethylangolensin (*O*-Dma), 1-(2,4-dihydroxyphenyl)-2-(4-hydroxyphenyl)-propan-1-one.

The method used was a modification of a method for determining the estrogen profile in urine by ion-exchange chromatography and capillary gas chromatography–mass spectrometry in the selected ion-monitoring mode (GC-MS-SIM, or GC/MS) (32–34). Originally, estrogens also were determined but because of very low concentrations of some fractions, the amount of urine saved for the purpose was too small and the analyses could not be repeated. Therefore, only the lignan and isoflavonoid values are presented. Only modifications of the method are described.

Protection of the carbonyl functions by ethoximation (necessary only for the estrogens), and extraction with a Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA) were carried out as described (33, 34). The removal of inhibitors of the enzyme hydrolysis by ion-exchange chromatography on a DEAE-Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden) column in the acetate form was done in a smaller column (0.5 × 3 cm instead of 0.5 × 5 cm). For hydrolysis and purification of the hydrolysate, before evaporation of the last fraction obtained from the above DEAE-Sephadex column, the following deuterated internal standards were added to the eluate: d_6-Enl and -End, d_4-Da and -Eq, and d_5-*O*-Dma (35, 36). This was followed by hydrolysis and Sep-Pak extraction; application of the methanolic extract directly on the QAE-Sephadex A-25 in the acetate form (0.5 × 5 cm); and elution of the estrogens, lignans, and Eq with 4 mL methanol as described. The modification in this step is that *O*-Dma and Da are eluted after this with 4 mL 0.2 mol acetic acid/L in methanol. This fraction is then, after evaporation of the solvent, ready for derivatization (trimethylsilyl ethers) and GC/MS. Selective fractionation of estrogens with vicinal *cis*-hydroxyls was carried out in a borate column with new dimension (0.5 × 3 cm instead of 0.5 × 2.5 cm). Elution of the diphenols was carried out as described and this fraction contains the isoflavan Eq and the two lignans Ent and End.

The two fractions containing lignans and isoflavonoid phytoestrogens and their deuterated internal standards are converted to their trimethylsilyl ether (TMS) derivatives (32) and quantified by GC/MS by using the following ion pairs (mass/charge): Eq, 386/390; Da, 398/402 (and 383/387); End, 410/416; Enl, 442/448; and *O*-Dma, 459/464 (36). The measurements were carried out with a Hewlett-Packard 5995 B GC/MS (Avondale, PA) instrument equipped with a Pascal work station and with an automatic injector.

Urinary excretion of < 0.0025 μmol/d cannot be measured, and between 0.0025 and 0.005 μmol/d the method must be regarded as semiquantitative. The mean values and interassay imprecision for the control pooled-urine sample, measured 59 times in single assays during 1 y, were as follows: Enl, 3.65 μmol/d (CV 7.4%); End, 0.364 μmol/d (CV 11.6%); and Eq, 0.042 μmol/d (CV 9.4%). For Da at a concentration of 0.028 μmol/d, the interassay imprecision is 11.0% (n = 14) and for *O*-Dma at the high concentrations in this study, the interassay imprecision is 8–10% (CV).

The samples were analyzed in two batches and the values for the control sample were almost identical both times and the same as in analyses before and after these two batches.

Statistical methods

The food data are presented as arithmetic means (±SD) and the lignan and phytoestrogen results as arithmetic means (±SD) and geometric means. Geometric means were used when necessary because of skewness of the distribution of the results. The statistical analyses were carried out by using the *StatView* program for Macintosh (Abacus Concepts, Berkeley, CA). The degree of univariate associations between two variables were estimated as Pearson's correlation coefficients (r). The pairs of

TABLE 1
Intake of various food stuffs by the Japanese women and men consuming a traditional Japanese diet*

Nutrient	Women (n = 10)	Men (n = 9)
	g/d	
Rice	578.5 ± 222.5	764.7 ± 240.3
Wheat	59.5 ± 46.0	139.0 ± 113.6
Potato	62.6 ± 30.2	55.2 ± 34.6
Sugar	8.1 ± 7.0	8.1 ± 7.4
Fats	13.1 ± 7.6	12.7 ± 6.9
Pulses and beans	56.5 ± 36.0	40.9 ± 32.0
Fruit	228.2 ± 111.9	146.9 ± 114.0
Green and yellow vegetables	60.6 ± 33.3	55.7 ± 35.2
Other vegetables	139.3 ± 69.3	130.9 ± 77.2
Pickles	32.9 ± 24.9	23.2 ± 21.2
Algae	1.8 ± 2.0	0.7 ± 0.7
Fish	98.7 ± 46.6	113.6 ± 56.5
Meat	37.0 ± 30.1	73.6 ± 58.4
Eggs	38.4 ± 16.6	57.4 ± 30.6
Milk	112.7 ± 131.0	90.9 ± 90.2
Beer	5.1 ± 16.1	454.6 ± 647.1

* $\bar{x}$ ± SD.

adjusted group means for the two groups studied (women and men) were compared by nonpaired t test.

Results

The intake of various types of food are shown in Table 1, and Table 2 shows the results of the calculations with regard to energy;

TABLE 2
Energy intake, intake of various nutrients, and some ratios in the two study groups*

Nutrient	Women (n = 10)	Men (n = 9)
Energy		
(MJ/d)	8.29 ± 1.64	10.79 ± 3.48
(kcal/d)	1973 ± 391	2569 ± 829
Animal protein (g/d)	35.3 ± 13.9	47.8 ± 18.9
Vegetable protein (g/d)	38.2 ± 10.1	45.1 ± 10.6
Total protein (g/d)	73.6 ± 12.2	93.0 ± 28.4
Carbohydrates (g/d)	311.4 ± 77.0	383.3 ± 100.6
Total fat (g/d)	44.4 ± 14.4	51.0 ± 25.9
Total fiber (g/d)	16.9 ± 4.9	15.3 ± 6.0
Animal protein (%)†	47.2 ± 15.9	49.8 ± 7.9
Proteins (%)‡	15.2 ± 2.1	14.6 ± 1.5
Carbohydrates (%)‡	64.6 ± 6.8	68.2 ± 5.1
Fats (%)‡	20.3 ± 5.5	17.2 ± 4.9
Fat (g/kg body wt)	0.86 ± 0.31	0.85 ± 0.37
Fiber		
(mg/J)	2.1 ± 0.7	1.5 ± 0.7
(g/1000 kcal)	8.8 ± 3.0	6.4 ± 3.0
Fiber (g/kg body wt)	0.33 ± 0.10	0.26 ± 0.09
Fat-fiber ratio	2.5 ± 0.9	2.4 ± 0.9

* $\bar{x}$ ± SD.
† Percent of total protein.
‡ Percent of energy.

TABLE 3
Dietary intake of soy products by the two groups studied*

Soy product	Women (n = 10)	Men (n = 9)
	g/d	
Tofu (soybean curd)	25.0 ± 22.9	18.7 ± 28.8
Miso (bean paste)	12.5 ± 6.2	8.5 ± 6.4
Aburaage (fried thin tofu)	2.6 ± 3.6	3.7 ± 4.2
Atuage (fried thick tofu)	4.0 ± 12.7	0.8 ± 2.3
Koridofu (dried soybean curd)	0.37 ± 0.78	0.07 ± 0.2
Fermented soybeans	2.4 ± 4.5	0.9 ± 2.8
Boiled beans	7.7 ± 17.8	6.5 ± 7.8
Soy sauce	22.9 ± 6.1	19.2 ± 4.7
Soy products (sauce excluded)	54.4 ± 34.3	39.2 ± 36.4

* $\bar{x}$ ± SD.

animal and vegetable protein; total proteins, carbohydrates, fats, and fiber; percentage animal protein and percentage protein; and carbohydrate and fat as percent of total calories. Furthermore, we calculated the fat intake per kilogram body weight, fiber intake per J (per 1000 kcal), and the fat-fiber ratio (Table 2). The diet was a low-fat (fat 17.2% and 20.3% of total calories for men and women, respectively), low-animal-protein diet with moderate amounts of fiber and a low fat-fiber ratio, which is typical for the traditional Japanese diet (37).

Table 3 shows the dietary intake of soy products, which were expected to be the most important source of precursors for the urinary isoflavonoids (3).

Table 4 shows the mean excretion values for the two lignans and three isoflavonoid phytoestrogens. The results show a relatively low excretion of enterolactone, a normal excretion for enterodiol, and a very high excretion of isoflavonoid phytoestrogens. The individual results showed large variation, particularly for equol (from 0 to 10.95 μmol/d). For comparison note that the geometric mean values in young omnivorous women living in Helsinki and in Boston for enterolactone, enterodiol, daidzein, equol, and O-desmethyl-angolensin were 2.46, 0.20, 0.22, 0.10, 0.03, and 2.05, 0.28, 0.32, 0.07, and 0.03 μmol/d, respectively (2).

TABLE 4
Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese women and men consuming traditional Japanese diet*

Compound	Women (n = 10)	Men (n = 9)
	μmol/d	
Enterolactone	1.4 ± 1.4 (0.89)	1.1 ± 0.7 (0.89)
Enterodiol	0.7 ± 1.3 (0.41)	0.4 ± 0.3 (0.22)
Total lignans	2.1 ± 2.6 (1.38)	1.5 ± 0.9 (1.13)
Daidzein	2.6 ± 4.0 (2.55)	2.2 ± 2.0 (1.45)
Equol	2.6 ± 4.0 (0.56)	3.0 ± 4.6 (0.54)
O-desmethylangolensin	0.7 ± 0.6 (0.51)	0.2 ± 0.3 (0.11)
Total isoflavonoids	6.9 ± 6.8 (4.73)	3.9 ± 3.3 (2.57)
Total diphenols	9.1 ± 9.3 (6.7)	5.4 ± 4.0 (4.1)

* $\bar{x}$ ± SD (geometric $\bar{x}$).

Table 5 presents a correlation matrix of various food components and urinary excretion of lignans and isoflavonoids in the total material of 19 subjects for whom both food and phytoestrogen data were available.

Discussion

In a previous study of oriental immigrant women from southeast Asia residing in Hawaii (38), the diet was similar to that consumed by the men and women in the rural village in Japan. In the present study the women had a greater energy intake (an additional ~2.1 MJ/d, or 500 kcal/d), which may be due to a physically more active life. However, the percentage intake of calories as fat and the dietary fiber and fat-fiber ratio were very similar to the corresponding values in the previous study. Except for the energy intake the values are very different from those seen in Western societies where the fiber intake is similar but the fat-fiber ratio is much higher. Women living in the Boston area had a fat-fiber ratio of 7.7 for the premenopausal women and 4.6 for the postmenopausal women compared with 2.5 for the women in the present study (39).

With regard to protein intake, expressed as g/d and as percentage of calories, the mean values in the present study were similar and slightly lower, respectively, than those of the immigrants from southwest Asia (38).

Our results are in good agreement with those from an earlier study of 300 female agricultural workers from 18 regions in Japan (37) except for dietary fiber intake, which was much lower (between 5 and 6 g/d) in the women in the earlier study (which may represent crude fiber intake). However, according to the national nutrition survey in Japan, the dietary fiber intake was 22.8 g/d in 1951 and decreased year by year to 17.4 g/d in 1985. These figures are in better agreement with our results obtained in 1985, which show a mean dietary fiber intake in the whole group of ~16 g/d. This latter value is also in good agreement with the value of 13 g/d for nonstarch polysaccharides found by analyses of the Japanese diet in another study (40). On the basis of these investigations and the present investigation, it may be concluded that the amount of dietary fiber in a traditional oriental diet is comparable with that in many Western societies (38–40). We may also conclude that the diet of our subjects was typical for a rural area, where the people to a large extent consume their own products and have a traditional Japanese diet.

The urinary excretion of Enl was, with few exceptions, low in both men and women (Tables 4 and 1A) and was the same as found for the postmenopausal breast-cancer patients in Boston (20). We found a weak correlation between intake of green and yellow vegetables and excretion of Enl and total lignans (Table 5) but no correlation with rice intake. Because these subjects consumed large amounts of rice, it seems justified to conclude that refined rice contains very low amounts, if any, of lignan precursors. There was a better correlation with the intake of soybeans, which thus also may be a source of Enl precursors (Table 5). It is known that soy sauce contains coniferyl alcohol the building block for lignans and lignin (41). The excretion of the lignan End was also found to be associated with the intake of beans and pulses and soy products in general (Table 5).

The excretion of the isoflavonoid phytoestrogens is very high in these Japanese men and women compared with values obtained in women living in Boston (2, 20) and in the Helsinki area (2, 18). The Japanese women in the present study excrete 10 times more Da and 20–30 times more Eq and *O*-Dma than did omnivorous and lactovegetarian women living in the above-mentioned two cities. Of the 19 subjects, 47% and 89% excrete micromole amounts of Eq and Da per day, respectively, a phenomenon very rarely seen in subjects consuming a Western diet but seen in subjects consuming a macrobiotic diet (2). The values in an additional study group of nine subjects, including three children (*see* Appendix A), were not significantly different from those in the two main groups (Tables 4 and 1A); they were in fact surprisingly identical. The excretion of matairesinol, the precursor lignan for enterodiol, was very low, but genistein excretion was very high. Genistein is the center of interest in many laboratories because of its very interesting antiproliferative and

TABLE 5
Correlation matrix of various food components and urinary excretion of ligans and isoflavonoids in the whole material (n = 19)

Nutrient	Enterolactone	Enterodiol	Total lignans	Daidzein	Equol	*O*-Desmethylangolensin	Total isoflavonoids	Total diphenols
Green and yellow vegetables	0.525*		0.460*					
Pulses and beans		0.541*	0.492*	0.679†	0.737†	0.617†	0.668†	0.693†
Algae				0.561*			0.450‡	0.430‡
Total fat					0.584†			
Percent fat calories					0.469*			
Fat-fiber ratio					0.507*			
Meat					0.507*			
Soy products (not sauce)		0.481*		0.583†	0.746§	0.601†	0.585†	0.588†
Boiled soybeans	0.758§	0.892§	0.849§	0.632†	0.693§		0.757§	0.801§

* $P < 0.05$.
† $P < 0.01$.
‡ $0.05 < P < 0.10$.
§ $P < 0.001$.

antimitogenic effects (*see* below); genistein showed the highest concentration of all phytoestrogens in urine in these nine subjects. The mean value was almost 6 μmol/d and a value as high as 15.5 μmol/d was observed. Also in this smaller group most variation in the excretion values was found for Eq (from 0.01 to 9.16 μmol/d). In 21.4% of all subjects, equol excretion did not significantly differ from zero: this group included two of the three children; the mother of these two children did not excrete equol in significant amounts.

The low excretion of Enl in the Japanese subjects compared, eg, with Finnish women (2), is most likely due to low intake of grain (whole-grain) products such as bread (2, 17, 18, 42, 43). The precursors of the mammalian lignans seem to be located in the aleuronic layer of the grain close to the fiber (15) but definite evidence for this location has not yet been obtained. The mean End values are similar to those observed in lactovegetarian American and Finnish women and higher than in the omnivorous women from the same countries (2, 20). It is likely that the majority of the lignans in these Japanese subjects is derived from nongrain plant products (pulses and beans), as suggested by the correlations found in Table 5.

Eq excretion correlated positively with the intake of total fat ($P < 0.01$), fat-fiber ratio ($P < 0.05$), and meat ($P < 0.05$) and deviated in this aspect from all the other isoflavonoids. Some subjects are not able to produce Eq at all, as also shown previously for nonJapanese subjects (44). It is possible that those consuming more fat and meat have an intestinal flora more capable of producing Eq from Da, known to occur in large amounts in soybeans (45). Algae may also be a source of isoflavonoids because a positive correlation was found with Da ($r = 0.56$; $P < 0.05$) and total isoflavonoids ($r = 0.45$; $0.05 < P < 0.10$, NS). Algae were suggested to contain factors protective against breast cancer (46).

Lignans and bioflavonoids are candidates for a role as cancer-protective agents (2, 14–16) and as steroid competitors for various enzymes (47). Enl inhibits the aromatase enzyme and competes with the natural substrate androstenedione for the binding site on the cytochrome P450 enzyme (H Adlercreutz, C Bannwart, LE Vickery, et al, unpublished observations, 1985). Phytoestrogens and lignans (48; H Adlercreutz, Y Mousavi, J Clark, et al, unpublished observation, 1987) show interaction with estrogen receptors and flavonoids have antiproliferative effects on the human-breast-carcinoma cell line ZR-75-1 (49). Genistein is a very specific inhibitor of the tyrosine-specific protein kinases (50–55) and platelet-activating-factor–stimulated platelet aggregation, phospholipase C, and tyrosine kinase activity (56). Tyrosine kinase is an important mediator of the effects of some biologically important growth factors such as epidermal growth factor, insulin, platelet-derived growth factor, and insulin-like growth factor on cells. The flavonoids and lignans bind to the type II estrogen-binding sites (15, 57), now also called the bioflavonoid receptor (47, 58), and may in this way regulate by inhibition cell growth and proliferation of hormone-dependent cancers (58). Enzymes metabolizing bioflavonoids and steroids show structurally close similarity (47), indicating that they have the same origin. Furthermore, the isoflavonoid coumestrol complements, as does estradiol, the topography of spaces between base pairs in unwound DNA and simultaneously hydrogen-bond phosphate moieties on opposite strands (59).

One of the most important biological effects of the lignans and isoflavonoids seems to be their stimulation of SHBG synthesis in the liver (2, 14, 16–18). A high SHBG concentration leads to decreased metabolic clearance rate for the sex hormones and lower biological activity. However, Japanese and British women were found to have the same SHBG total-binding capacity, even though Japanese women bound relatively more estradiol to SHBG. This was suggested to be a result of lower affinity of albumin for estradiol in these women (60). It is possible that the phytoestrogens in the high amounts occurring in Japanese women could compete with estradiol for the albumin-binding sites and in this way lead to relatively more binding to SHBG.

SHBG concentrations tend to be lower in breast-cancer patients, particularly in postmenopausal women, and this seems at least partly to be due to diet (15). SHBG-binding capacity was significantly smaller in postmenopausal but not in premenopausal Japanese subjects with breast cancer compared with Japanese control subjects (61), agreeing with our own more recent results in American postmenopausal (43) women. Finnish premenopausal women with breast cancer did not differ in this respect from omnivorous control subjects but they had lower SHBG than did lactovegetarian women (18). Diet seems to be a much more important risk factor for postmenopausal than for premenopausal breast cancer (15). Miso (Japanese soybean paste) (62) or powdered soybean chips (63) (both before and after denaturation of the protease inhibitors) showed a tendency to decrease mammary-tumor formation and growth rate in rat breast-cancer models and soybean diet also reduced breast-tumor incidence in irradiated rats (64). This agrees with the slower average growth rate of postmenopausal breast cancers in Japanese compared with caucasian women in Hawaii (65).

The high concentration of phytoestrogens in the urine of Japanese men could be protective with regard to prostate cancer. Both lignans and isoflavonoids have estrogenic effects in numerous biological systems and may, because of this property, inhibit development of prostatic cancer. It is well known that in Japan and some other Asian countries, despite the same incidence of latent small or noninfiltrative prostatic carcinomas as in Western societies, the mortality is low (25–27). The high exogenous phytoestrogen concentrations could inhibit the growth of the latent carcinomas, postponing their development and making it more likely that the subjects die from some other disease (theory proposed in 1985) (66). Furthermore, the inhibitory effect of genistein on tyrosine-specific protein kinases of certain growth-factor receptors could play an important role. Decreased risk of prostate cancer is seen in Seventh-day Adventist men (67) consuming much beans, lentils, and peas and some dried fruits (rich sources of bioflavonoids) and in men of Japanese ancestry in Hawaii consuming much rice (mainly starch, which has some fiber-like effects in the gut) and tofu (68), supporting the view that these compounds are protective. Recently, Santti's group in Turku, Finland, in a collaborative study with us, observed that dietary soy prevented the development of precancerous changes in a neonatally estrogenized mouse used as a model for prostatic cancer (69), showing that dietary factors may already be important in the fetal and neonatal periods. This study and our observation of high phytoestrogen excretion in urine of children is important because they suggest that these compounds may change the endocrine milieu at the cellular level both in the neonatal period and in prepubertal and adolescent children. Thus, the results cited above and discussed more

extensively elsewhere (14, 15) speak for a role of the diphenols as cancer-protective substances.

It is concluded that Japanese subjects excrete very large amounts of isoflavonoids in urine, mainly genistein, daidzein, and equol, and that the lignan excretion is low. The high excretion of isoflavonoids in urine is related to the intake of soy products in the traditional Japanese diet.

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APPENDIX A

Additional experiments with a modification of the method

The method used in this study was modified further by including the determination of the plant lignan matairesinol [(3R-*trans*)-dihydro-3,4-*bis*[(4-hydroxy-3-methoxy-phenyl)methyl]-2(3H)-furanone)] (intraassay CV = 15.2% and interassay CV = 13.9%) and the isoflavonoid genistein (4',5,7-trihydroxyisoflavane) (intraassay CV = 4.5% and interassay CV = 11.6%) in the assay (1). Because further samples from the present study were not available and because of the recent great interest in genistein we used this new assay in nine other Japanese subjects (three men, three women, and three children) living in Kyoto and consuming a traditional Japanese diet before and during the 24-h urine collection.

TABLE 1A
Urinary excretion of lignans and isoflavonoid phytoestrogens (μmol/d) in nine Japanese subjects (six adults, three children) living in Kyoto and consuming traditional Japanese diet during the urine collection period

Subject, sex, age	Matairesinol	Enterolactone	Enterodiol	Total lignans	Daidzein	Equol	*O*-Desmethylangolensin	Genistein	Total isoflavonoids	Total diphenols
1, M, 41 y	0.010	0.05	0.09	0.15	5.25	6.15	0.12	15.52	27.04	27.20
2, F, 33 y	0.003	2.44	0.15	2.59	3.11	0.01	0.98	4.48	8.58	11.17
3, M, 7 y	0.003	0.07	0.09	0.16	3.23	0.01	0.06	5.66	8.97	9.13
4, M, 6 y	0.006	2.24	0.68	2.93	2.15	0.85	0.51	3.41	6.93	9.85
5, M, 8 y	0.007	0.04	3.39	3.43	3.02	0.02	0.81	4.80	8.64	12.07
6, F, 42 y	0.006	3.25	0.25	3.50	2.20	0.16	1.17	3.55	7.07	10.58
7, M, 38 y	0.012	0.70	0.25	0.96	1.60	0.07	0.40	4.93	6.99	7.95
8, M, 26 y	0.019	1.94	0.18	2.13	3.38	9.16	0.23	7.99	20.76	22.89
9, F, 30 y	0.005	0.62	0.25	0.88	1.25	3.28	0.21	1.85	6.60	7.47
$\bar{x}$	0.010	1.26	0.59	1.86	2.8	2.19	0.50	5.80	11.29	13.15
Geometric $\bar{x}$	0.010	0.50	0.27	1.17	2.58	0.25	0.35	4.91	9.81	11.89

Table 1A shows the individual urinary lignan and isoflavonoid excretion in the additional three men, three women, and three children studied by the new modified procedure, including the results of assays for matairesinol and genistein.

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1. Adlercreutz H, Fotsis T, Bannwart C, Wähälä K, Brunow G, Hase T. Isotope dilution gas chromatographic-mass spectrometric method for the determination of lignans and isoflavonoids in human urine, including identification of genistein. Clin Chim Acta 1991;199:263–78.

Increasing use of soyfoods and their potential role in cancer prevention

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Abstract The United States produces approximately half of the world's soybeans. Although most of what is produced is used as animal feed, soy-protein products (eg, soy-protein flour, concentrates, and isolates) are used extensively by the food industry, primarily for their functional characteristics, such as emulsification. During the past decade, however, there has been a marked increase in the use of both traditional soyfoods, such as tofu and soymilk, and second-generation soyfoods, products which generally simulate familiar American dishes. Recently, attention has focused on the possible role of soybean consumption in reducing cancer risk. Soybeans contain, in relatively high concentrations, several compounds with demonstrated anticarcinogenic activity. Two of these compounds—protease inhibitors and phytic acid—have traditionally been viewed as antinutrients. The scientific community has begun to appreciate the potential importance of nonnutritive dietary compounds (phytochemicals) in foods such as soybeans. Dietitians need to become more aware of the phytochemical content of foods and the possible effect of phytochemicals on health and disease. *J Am Diet Assoc. 1991; 91:836-840.*

The United States produced more than $10 billion of soybeans in 1989, about half the world's total (1). Although most of the soybeans produced are used as animal feed (1), soy-protein products have been used extensively by the food industry since 1957 (2). Furthermore, during the past decade, there has been a marked increase in both the consumption of traditional soyfoods—such as tofu, soymilk, miso, and tempeh—and in the development of second-generation soyfoods (3,4). Second-generation soyfoods generally simulate traditional meat and dairy products, eg, soy hot dogs, soy sausage, and soy cheese.

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The increase in soyfood consumption, which is expected to continue throughout this decade, is attributed to a number of factors, including economics, health, ethics, and the environment. Recently, the potential role of soybeans in cancer prevention has received attention (5). As soyfoods continue to become part of the American diet, it is important for nutritionists and dietitians to familiarize themselves with soyfoods and their possible impact on health and disease.

Historical perspective

According to tradition, soybeans were one of five "sacred" crops named by Chinese emperor Sheng-nung nearly 5,000 years ago (6). The soybean has been widely consumed for hundreds of years throughout Asia. Soybeans were reportedly first brought to the United States in 1804 as ballast aboard a ship from China, but not until the 1890s was serious interest in soybeans expressed (7). World War I brought an increased interest in soybeans as a potential source of oil and of inexpensive, high-quality protein (7). In 1917, only 50,000 acres of soybeans were planted in the United States, but by 1931, this number had increased 70-fold (8). Throughout this century, US soybean production has steadily increased; soybeans are now the second most important cash crop in the United States (9).

Soybeans and the US food supply

Soy-protein products

Soy protein products are grouped into three general categories: soy flour, soy-protein concentrates, and soy-protein isolates (2). The market for soy-protein products is estimated at around $200 million per year (3,4). More than 90% of the soybeans consumed by human beings in the United States, excluding soybeans used for soy oil, is in the form of soy-protein products (3). These products are made from defatted soybean flakes, range in protein content from about 50% to 90% and are added to a vast array of foods, primarily for their functional characteristics, such as emulsification (2). The per capita intake of soy-protein products in the United States has increased approximately 40% during the past 10 years, but soybean consumption is still less than 5 g/day per person (3). Thus, the nutrient contribution of soy-protein products for most individuals is negligible.

Two additional soy products used by the food industry are hydrolyzed vegetable protein and textured soy protein (2). Hydrolyzed vegetable protein is used as a whipping and flavoring agent and in the manufacture of soy sauce. Textured soy protein is frequently used as a ground beef extender, most notably by schools and the military. Textured soy protein is also used to produce meat analogues. The school lunch program uses an estimated 100 million lb textured soy protein annually (3).

Retail soyfoods sales

The US retail soyfood market has undergone considerable growth in recent years. In 1989 sales were more than $600 million, a fourfold increase since 1980 (3,4). During the past decade, approximately 200 new soyfood products have entered the market annually (3,4). After soy sauce, tofu is the biggest seller among all soyfoods, followed by second-generation soyfoods, soymilk, miso, soynuts, and tempeh (3).

Sales of soymilk and second-generation products are expected to increase substantially during the next 10 years (3). Soymilk consumption in particular is on the rise; sales have increased 40% during each of the last 2 years (3). By the year 2000, sales of soymilk are projected to increase sixfold and sales of second-generation products are expected to increase about fourfold (3). The increased consumption of soymilk has been attributed to improved taste, increased shelf-life through improved packaging, and lower cost resulting from increased domestic production.

The increasing number of second-generation soyfoods is particularly noteworthy. Since 1987, 552 new second-generation products have been developed; more than half of these contained tofu as their primary soy ingredient (3). Products range from main entrees—including frozen, shelf-stable, and refrigerated prepared foods—to meat alternatives such as tofu hot dogs and tempeh burgers, cheese alternatives, yogurts, and nondairy frozen desserts. In 1989, more than two thirds of all tofu and one third of all second-generation soyfoods were sold in supermarkets, a further indication that soyfoods are entering the mainstream (3). Nevertheless, with the exception of soy sauce, most soyfoods are consumed by a relatively small percentage of the population. For these individuals, soyfoods can be a major component of the diet.

Nutrient contribution of soyfoods

Whole soybeans are a good source of protein, fiber, calcium, iron, zinc, phosphorus, magnesium, thiamin, riboflavin, niacin, and folacin (10). Processing the whole soybean to make soyfoods affects the nutrient content of the resulting product (Table 1). Foods made from the whole soybean, such as tempeh, natto, and miso, are good sources of fiber and calcium, whereas soymilk and products made from soymilk, such as tofu and soy cheeses, are not. Calcium can be successfully added to soymilk (11); however, this has been done only sparingly, primarily in malted soy drinks. Calcium content of tofu varies quite notably. The values listed in Table 1 appear to be somewhat higher than one might surmise from a random, informal examination of commercially available products.

Sources of vitamin B-12 in soyfoods are an important consideration for certain individuals whose diet may be devoid of or contain only limited amounts of animal products. Some reports (10,12,13) indicate that fermented soyfoods, ie, miso and tempeh, contain vitamin B-12, either through design or by contamination, but other reports (14,15) suggest that most, if not all, of the vitamin B-12 in these products is in the form of analogues.

Soyfoods are relatively high in fat, but still may be lower in total fat than the foods they frequently replace, such as meats and cheeses. Soyfoods are certainly lower in saturated fat and cholesterol. Tofu ranges from about 35% to 50% fat on a caloric basis (silken tofu, which includes the whey, is generally lower in fat), whereas the fat content of soymilk varies depending on additional ingredients used. In contrast to skim milk, soymilks that are lower in fat are generally not produced by removing fat, but by adding carbohydrate, which is used as a sweetening or flavoring agent. Soybean oil is the only commonly consumed oil in this country that contains appreciable amounts of ω-3 fatty acids (α-linolenic acid). Consequently, soyfoods are a convenient means of obtaining ω-3 fatty acids from plant sources. However, hydrogenation of soy oil notably reduces linolenic acid content (16).

Soybeans and cancer risk

In 1929, soybean consumption was offered as a possible explanation for the greater stamina of northern Chinese compared with their rice-eating counterparts in the south (17). Throughout this century, soybeans have been the subject of considerable investigation covering a wide range of interests. Recently, the potential role of soybeans in cancer prevention has received attention. The contribution of soybeans to the diets of Oriental countries, such as Japan and China, has prompted some investigators to suggest that soybeans may contribute to the relatively low rates of breast and colon cancer in these countries. In June 1990, the National Cancer Institute held a workshop to examine this relationship (5).

Several compounds with anticarcinogenic activity are found in relatively high concentrations in soybeans. Among those thus far identified are isoflavones, protease inhibitors, phytic acid, saponins, phytosterols, and phenolic acids. Ironically, two of these compounds, protease inhibitors and phytic acid, have traditionally been viewed as antinutrients. Phytic acid effectively binds a variety of polyvalent metals, especially iron, and is considered to affect adversely the bioavailability of iron in soyfoods (18). The ability to chelate iron, however, may be responsible for the antioxidant and anticarcinogenic properties of phytic acid (19,20). Among the hypothesized anticarcinogens in soybeans, isoflavones and protease inhibitors are found in the highest concentrations relative to most other commonly consumed foods; consequently, in the discussion to follow, only these compounds will be examined, along with epidemiologic studies of soybean consumption and breast and colorectal cancer risk.

Experimental studies

Isoflavones. Barnes et al (21) showed that diets composed of as little as 5% (*wt/wt*) soybeans significantly inhibited chemically induced mammary cancer in rats. The study was particularly noteworthy because both raw and autoclaved soybeans were protective. This was an important observation because the protease inhibitors in

Table 1. Proximate composition and selected nutrient content of various soyfoods in common serving sizes and in 100-g edible portions (10)

component	miso[a]		natto[b]		okara[c]		roasted soybeans[d]		soymilk[e]		soy sauce (tamari)[f]		tempeh[g]		firm, raw tofu[h]		regular, raw tofu[h]	
	½ c	100 g	½ c	100 g	½ c	100 g	½ c	100 g	½ c	100 g	1 tsp	100 g	½ c	100 g	¼ B[i]	100 g	¼ B	100 g
water (g)	57	41	48.4	55	50	82	1.7	2.0	112	93	11.9	66	45.6	55	57	70	98	85
kcal	284	206	187	212	47	77	405	471	39	33	11.0	60	165	199	118	145	88	76
protein (g)	16.3	11.8	15.6	17.7	2.0	3.2	30.3	35.2	3.3	2.8	1.9	10.5	15.7	19.0	12.8	15.8	9.4	8.1
lipid (g)	8.4	6.1	9.7	11.0	1.1	1.7	21.8	25.4	2.3	1.9	0.02	0.1	6.4	7.7	7.1	8.7	5.6	4.8
carbohydrate (g)	38.6	28	12.6	14.4	7.7	12.5	28.9	33.6	2.2	1.8	1.0	5.6	14.1	17.0	3.5	4.3	2.2	1.9
crude fiber (g)	3.4	2.5	1.4	1.6	2.5	4.1	4.0	4.6	13.2	1.1	0.0	0.0	2.5	3.0	0.12	0.15	0.09	0.08
calcium (mg)	92	66	191	217	49	80	119	138	5	4	4	20	77	93	166[k]	205[k]	122[k]	105[k]
iron (mg)	3.8	2.7	7.6	8.6	0.79	1.3	3.4	3.9	0.7	0.6	0.43	2.38	1.9	2.3	8.48	10.5	6.2	5.4
zinc (mg)	4.6	3.3	2.7	3.0	...[l]		2.7	3.1	0.27	0.23	0.08	0.43	1.5	1.8	1.27	1.57	0.93	0.8
thiamin (mg)	0.13	0.1	0.14	0.16	0.01	0.02	0.09	0.1	0.19	0.16	0.01	0.06	0.11	0.13	0.13	0.16	0.09	0.08
riboflavin (mg)	0.35	0.25	0.17	0.19	0.01	0.02	0.13	0.15	0.08	0.7	0.27	0.15	0.09	0.11	0.08	0.9	0.60	0.05
niacin (mg)	1.19	0.86	0.0	0.0	...[l]		1.2	1.4	0.18	0.15	0.71	3.95	3.8	4.6	0.31	0.38	0.23	0.2
vitamin B-6 (mg)	0.3	0.22	...[l]		...[l]		0.18	0.21	0.05	0.4	0.36	0.2	0.25	0.3	0.08	0.09	0.06	0.05
folacin (μg)	45.5	33	...[l]		...[l]		182	211	1.8	1.5	3.3	18.2	43.2	52.0	23.7	29.3	17.4	15.0

[a]Cooked soybeans are dusted with *Aspergillus oryzae*, a bacterial starter, fermented, and pressed into a paste.
[b]Whole soybeans are steamed until soft, inoculated with *Bacillu natto*, fermented, and then commonly sold in 3- to 4-oz packages wrapped in straw.
[c]The pulp remaining after the soymilk is filtered; it is highly perishable.
[d]Whole soybeans are either dry- or oil-roasted in a pan or oven until crunchy.
[e]After overnight soaking, soybeans are pulverized, extensively cooked (to inactivate protease inhibitors), and then filtered resulting in a liquid soymilk.
[f]Insoluble dietary fiber as determined by the neutral detergent fiber method.
[g]Cooked soybeans are dusted with *Aspergillus oryzae*, shaped into koji nuggets, incubated, and mixed with salt and water to produce a mash called moromi. After aging, the moromi is pressed to yield a liquid and the oil is removed producing soy sauce.
[h]Cooked soybeans alone or with other grains are fermented with *Rhizopus oligosporus* producing a chunky-textured cake about ¾ inch thick.
[i]Soymilk is coagulated with a calcium or magnesium salt (nigari), the whey is discarded, and the curds are pressed to form a cohesive bond. The degree of pressing produces either soft, regular, or firm tofu. Calcium values refer to product made with nigari. The proximate composition of tofu shown here varies notably from recently adopted industry standards.
[j]B = block; one block refers to tofu 1¼ by 1⅝ by 1⅜ in.
[k]No value reported.

soybeans, which are thought to be potent chemopreventive agents, are destroyed by autoclaving (22). The data of Barnes et al suggested that the isoflavones in soybeans were responsible for tumor inhibition. Soybeans are one of the few commonly consumed foods containing appreciable amounts of isoflavones (23).

A considerable amount of isoflavone research has been conducted, although very little is cancer related. For many years, isoflavones have been known to cause reproductive problems in agricultural animals (24), although similar effects have not been observed in populations consuming soyfoods.

In vitro data indicate that several isoflavones are weak estrogens, having perhaps as little as 1/100 of the affinity for the estrogen receptor as estradiol-17B (25,26). In vivo, isoflavones may act as antiestrogens in the presence of high levels of endogenous estrogens (25). This may occur because the isoflavones bind to the estrogen receptor without eliciting a substantial estrogenic response and competively inhibit the more potent estrogenic agonists from binding. The hypothesized antiestrogenic effect of isoflavones may help to reduce breast and perhaps endometrial and ovarian cancer, as these cancers are thought to be estrogen dependent (27). However, the effects of isoflavones may not be limited solely to hormone-related cancers.

In vitro, genistein, one of the isoflavones present in soybeans, inhibits tyrosine protein kinases, DNA topoisomerases, and S6 kinases (28-31). The activity of these enzymes is enhanced in oncogene-transformed cells (28-31). Consequently, isoflavones may have a role to play in the prevention of a wide range of cancers.

Human studies suggest that isoflavones may be physiologically important. Setchell et al (32) found that consumption of textured soy protein increased urinary isoflavone levels as much as 1,000-fold, although there were marked differences in isoflavone metabolism among the subjects in this study. Similarly, Adlercreutz et al (33) reported that urinary isoflavone levels were about 30 times higher in Japanese women consuming a traditional diet than in Finnish women.

Several studies have looked specifically at the estrogenic/antiestrogenic effects of soybeans. In postmenopausal women, soy intake appears to produce slight estrogenic effects (5,34); a recently completed study (A. Cassidy, S. A. Bingham, K. D. R. Setchell, unpublished data, 1991) however, found that in premenopausal women soy ingestion significantly lengthened the menstrual cycle. These studies suggest that isoflavones possess both antiestrogenic and estrogenic activity and, in premenopausal women, soy consumption influences hormonal patterns in a manner potentially protective against breast cancer.

Protease inhibitors. For almost 50 years, the protease inhibitors in soybeans have been known to affect protein utilization adversely (35). Ironically, protease inhibitors were one of the first identified compounds to prevent

promotion of experimentally induced breast and colon cancer (22). The primary protease inhibitors in soybeans are the Kunitz trypsin inhibitor and the Bowman-Birk trypsin and chymotrypsin inhibitor (BBI). Most, but not all protease inhibitor activity is destroyed upon heating. Concern has been expressed over the potential harmful effects of consuming foods high in protease inhibitor content, particularly in the case of soy-based infant formulas (36). Raw soybean consumption causes pancreatic hypertrophy in rats (37). However, the response of the pancreas to protease inhibitors differs markedly among species (38); in populations consuming cooked soybean products no adverse effects have been observed.

Consumption of raw soybeans, which contain considerable protease inhibitor, markedly reduced experimentally induced breast cancer, although soy-fed rats weighed significantly less than the controls (39). Chymotrypsin inhibition is thought to be responsible for the anticancer activity of soybean protease inhibitors, although the inhibition of other proteases may also be important for inhibiting cancer (40). Soybean extracts containing BBI and/or pure BBI have been shown to inhibit experimental colon (41), lung (42), and oral cancer (43). Additionally, BBI inhibits x-irradiation–induced c-myc expression (44), decreases H_2O_2 formation by activated human polymorphonuclear leukocytes (45), and the soybean trypsin inhibitor suppressed the promotional effects of 12-0-tetradecanoylphorbol-13-acetate on transformation in C3H10T1/2 cells (40). Participants at a recent workshop expressed considerable enthusiasm for the development of protease inhibitors as chemopreventive agents (22).

Epidemiology. Much of the current thinking about diet-cancer hypotheses is based on studies comparing dietary intake among countries with the international variation in cancer incidence. These types of data are not available for soybean consumption and are probably not feasible to obtain. Most countries consume only minor amounts of soy products and/or soyfoods; only a handful, such as Taiwan, Japan, North and South Korea, Indonesia, and Hong Kong consume appreciable amounts (46). The epidemiologic data on soy and cancer consist primarily of case-control studies; case-control studies generally have been less supportive of diet-cancer associations.

Breast cancer. Despite the experimental data on isoflavones, little epidemiologic evidence suggests that soybeans lower breast cancer risk. Although a recent case control study (47) is strongly supportive of a protective effect of soy, Phillips (48) studied Seventh-day Adventists in the United States and found no difference in the consumption of vegetarian protein products between case and control subjects (48). However, vegetarian protein products were not exclusively soy, but included gluten-based products as well. No specific data on soy intake were recorded. Hirohata et al (49) found that soy was unrelated to breast cancer risk but, again, grams of fat derived from soy products rather than soy intake were recorded.

A very large prospective study involving 29 health districts and almost 143,000 women in Japan also found bean consumption to be unrelated to breast cancer mortality (50). No description of what constituted bean consumption was provided; however, from other dietary data it is apparent that bean consumption would primarily refer to soy products. In contrast, Nomura et al (51) found that soyfood consumption reduced breast cancer risk. However, findings were based on the dietary intake of husbands of subjects. Nomura and colleagues assumed that the dietary patterns of husbands and wives were similar. Spouses of controls consumed about 60% more miso soup ($P<.05$) and about 30% more tofu ($P<.16$) than did spouses of cases. However, Hiriayma (52) cautions that miso soup is frequently eaten in conjunction with vegetables, a factor that could be at least partially responsible for any protective effects of miso. Finally, Lee et al (47) in a case-control study of diet and breast cancer in Singapore found that soy protein in premenopausal women was protective ($P=.02$) and suggested that the phytoestrogens in soy may be responsible for this effect.

It may not be appropriate to evaluate soybeans on nutrient content alone; dietitians need to know about the nonnutritive dietary compounds, called phytochemicals, which may have anticarcinogenic effects

Colorectal cancer. Several studies suggest that soy consumption may lower colorectal cancer risk. Phillips (48) reported that colon cancer risk was reduced in individuals frequently consuming vegetarian protein products, although the effect was not significant (relative odds = 0.4). Similarly, Watanabe et al (53) found that frequent consumption of soybeans and tofu markedly decreased both rectal and colon cancer risk (relative odds = 0.14 for rectal cancer and 0.63 for colon cancer). Tuyns et al (54) also found that frequent consumption of soybeans decreased both rectal and colon cancer risk ($P<.0001$), and Poole (55) found that frequent consumption of tofu decreased colon cancer risk by approximately one half.

In contrast, Tajima and Tominaga (56), reported that consumption of miso soup significantly increased rectal cancer risk (relative risk, 2.05; $P<.05$) in Japanese subjects, although tofu only mildly increased risk. However, tofu was not associated with colon cancer risk in this study (55) and consumption of miso soup reduced colon cancer risk by one half.

Conclusions.

Overall, the epidemiologic data suggest that soy consumption may lower colorectal cancer risk, whereas there is only moderate support for a role of soy in reducing breast cancer; however, relatively little work has been conducted in this area. Experimental work indicates that soy contains several potential anticarcinogens and that both estrogenic and antiestrogenic responses have been

observed in human beings consuming soy. Further investigation of the relationship between soy and cancer is needed.

Implications

As soyfoods continue to enter the American mainstream diet, their nutritional contributions will become increasingly important. However, it may no longer be appropriate to evaluate many foods, such as soybeans, solely on the basis of their nutrient content. The scientific community has begun to appreciate the effect of nonnutritive dietary components (phytochemicals) on health and disease, including diseases such as cancer. This places an additional burden on the nutrition community to become more aware of the phytochemical content of foods. Notably reducing the incidence of diet-related cancers will require major changes in the American diet. The extent to which soyfoods may help to achieve this goal remains to be seen.

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(54) Title: DIETARY SUPPLEMENT AND METHOD FOR THE TREATMENT OF MENOPAUSE AND MANIFESTATIONS OF AGING

(57) Abstract

The present invention provides a dietary supplement containing a mixture of lipids, extracted from natural sources, which has a beneficial effect on a variety of disorders and syndromes in humans. The dietary supplement of the present invention is preferably formulated as a beverage, but may be formulated in granule, capsule or suppository form. The dietary supplement of the present invention provides a combination of phospholipid and neutral lipid supplements to the diet. This dietary supplementation alleviates to a great extent the occurrence and severity of the hot flush syndrome in menopausal women. Furthermore, these supplements slow the deterioration of mental processes and to markedly improve memory, especially short-term memory, of individuals who have taken the supplement of the present invention.

DIETARY SUPPLEMENT AND METHOD FOR THE TREATMENT OF MENOPAUSE AND MANIFESTATIONS OF AGING

Field of the Invention

This invention relates to compositions comprised of natural ingredients which exhibit a pronounced beneficial effect on certain conditions including reducing the intensity and frequency of menopausal hot flushes. Compositions of the present invention also have been shown to enhance the memory in humans and to reduce certain adverse effects on human mental processes that are associated with the aging process.

This invention also relates to methods for reducing the intensity and frequency of menopausal hot flushes, treating vaginal dryness and enhancing the memory and mental processes of humans, which have been diminished during the aging process, by administering the compositions of the present invention.

Background of the Invention

The aging process is known to deteriorate certain body functions; among these, the ability of women to produce ova for reproduction. Associated with the deterioration of ova production is estrogen withdrawal and vaginal dryness. The hot flush is a physiological response to estrogen withdrawal which often accompanies deteriorating reproductive function during menopause. Hot flushes are associated with a hormonal imbalance and result from tissue aging.

The hot flush is common to about 75% of menopausal women, and usually occurs in women between the ages of 45 and 55. About 30% of all women in this age group suffer from severe flushes for a certain period of time ranging from about 6 months to about 5 years. Five percent (5%) of all women continue to suffer from the syndrome for the remainder of their lives.

Estrogen treatment is effective in many cases, but there is controversy amongst physicians as to the duration and the side effects of such treatment. Presently, there is no alternative treatment for relieving hot flush syndrome in women other than estrogen treatment.

Other body functions, including those associated with mental processes and especially memory, also deteriorate with age. The loss of certain mental processes differs from one

individual to another within the same age group. It is estimated that about 50% of people over the age of 65 suffer from a certain degree of memory loss, and about 15% of those people suffer from a severe degree of such mental dysfunction. It is a very distressing syndrome and little can be done to alleviate the deterioration.

It is an object of the present invention to provide novel dietary supplements which may be taken to alleviate the occurrence and severity of hot flushes in women and to improve the memory and mental processes of individuals.

It is an additional object of the present invention to provide a method for alleviating the occurrence and severity of hot flushes in women which are associated with hormonal imbalance and tissue aging.

It is a further object of the present invention to provide a method for improving memory, especially short-term memory and other mental dysfunction associated with the aging process.

Brief Description of the Invention

The present invention provides a dietary supplement composition containing a mixture of lipids, extracted from natural sources, which has a beneficial effect on a variety of disorders and syndromes in humans. The dietary supplement of the present invention is preferably formulated as a beverage, but may be formulated in granule, capsule or suppository form.

The dietary supplement composition of the present invention provides a combination of phospholipid and neutral lipid supplements to the diet. This dietary supplementation alleviates to a great extent the occurrence and severity of the hot flush syndrome. Furthermore, these supplements slow the deterioration of mental processes and markedly improve memory, especially short-term memory, of individuals who have taken the supplement of the present invention.

The lipid supplement of the present invention is a mixture of phospholipids and glycerides from animal and plant sources having a defined composition profile of fatty acids and phosphlipid head groups which are essential for proper effectiveness.

In relevant part, compositions of the present invention comprise:

a) a lipid mixture obtained from egg yolks, said lipid mixture containing a mixture of phospholipids comprising about 5% to about 40% by weight of the total weight of phospholipids and neutral lipids in said supplement;

b) a phospholipid mixture obtained from a source other than egg yolks in an amount equal to about 5% to about 40% by weight of the total weight of phospholipids and neutral lipids in said supplement wherein at least about 30% by weight of the total phospholipid content of said phospholipid mixture is comprised of negatively charged phospholipids;

c) a neutral lipid mixture obtained from a source other than egg yolks in an amount equal to about 20% to about 80% by weight of the total weight of phospholipids and neutral lipids in said supplement, and wherein said lipid mixture, said phospholipid mixture, and said neutral lipid mixture together comprise at least about 5% by weight of said supplement, said negatively charged phospholipids comprise at least about 10% by weight of said phospholipids and neutral lipids in said supplement and the weight ratio of neutral lipids to phospholipids in the supplement ranges from about 1:1 to about 2.5:1.

These dietary supplement compositions may be further comprised of antioxidants, and formulated with alcohol as beverages for ingestion. Certain additives, for example flavorings and/or coloring agents, may be added to make the supplement more palatable or to enhance the nutritional value of the supplement. Diluents and excipients may be added to the compositions of the present invention to produce a dietary supplement that may be used in capsule, granule or suppository form.

Compositions of the present invention are useful as dietary supplements for alleviating the occurrence and severity of hot flushes in menopausal women. In addition, these compositions are effective as dietary supplements for slowing, and in certain cases, reversing the deterioration of mental processes associated with the process of aging.

The present invention also relates to methods for alleviating the occurrence and severity of hot flushes in women which are associated with hormonal imbalance during and after menopause and tissue aging.

The present invention further relates to a method for improving memory, especially short-term memory and other mental dysfunction associated with the aging process.

Detailed Description of the Invention

Dietary supplement compositions of the present invention comprise a lipid mixture obtained from egg yolks, a phospholipid mixture obtained from a source other than egg yolk, preferably having a high percentage of negatively charged phospholipids, and a neutral lipid mixture obtained from a source other than egg yolks, preferably containing a high percentage of neutral lipids. Preferred embodiments additionally comprise an effective amount of a physiologically acceptable antioxidant and/or at least about 0.5% of ethyl alcohol.

The lipid mixture is a mixture of phospholipids and neutral lipids obtained from egg yolk by extraction. The lipid mixture may be obtained by extracting egg yolks with any number of non-polar and/or polar solvents including chloroform, methylene chloride, acetone, chloroform-methanol 1:1 v/v, tetrahydrofuran, diethyl ether, diethyl ketone, acetonitrile, methanol and ethanol, among others, followed by precipitation of a certain fraction of the extracted lipid, preferably with acetone. Such a procedure, described in U.S. Patent No. 4,474,773, which is hereby incorporated by reference, produces a lipid mixture of phospholipids and neutral lipids wherein the percentage of neutral lipids is relatively high compared to the phospholipid component (weight ratio of neutral lipids to phospholipids generally ranges from about 1:1 to about 7:3). AL-721™ (Ethigen Corporation, U.S.A.), described in U.S. Patent No. 4,474,773, is an example of a commercially available product produced using such a procedure.

Alternatively, and preferably, the lipid mixture obtained from egg yolk contains a high percentage of phospholipid compared to neutral lipid. In order to produce such a lipid mixture, it is preferable to extract the egg yolks with a polar solvent, preferably boiling ethyl alcohol, for a period of about one hour. It is preferred that 95% ethanol be used, but an ethanol-water mixture containing at least about 70% ethanol may also be used to effect the extrac-

tion. Other polar solvents may be used, but preferred solvents include those which are pharmaceutically compatible and are non-toxic in small amounts. The extraction of egg yolks with a polar solvent produces a mixture of phospholipids and neutral lipids in which phospholipids, more polar than the neutral lipids, predominate. Preferably, the weight ratio of phospholipids to neutral lipids in the lipid mixture obtained from egg yolk is about 1:2.

The lipid mixture obtained from egg yolk comprises about 5% to about 40%, and preferably, about 10% to about 30% by weight of the total weight of phospholipids and neutral lipid sin the compositions. The amount of lipid mixture obtained from the egg yolks generally equals about 5% to about 15% by weight of the total weight of crude egg yolk.

Compositions of the present invention also comprise a phospholipid mixture obtained from a source other than egg yolks containing a mixture of phospholipids wherein at least about 30% and preferably at least about 50% of the phospholipids are negatively charged phospholipids. Any number of non-egg yolk sources of phospholipids are available including, for example, vegetable or soya lecithin (commercial granulated soya lecithin) and brain phospholipids. The phospholipid source is selected such that at least 30% by weight of the phospholipids are negatively charged phospholipids. Negatively charged phospholipids are those phospholipids that have a net negative charge and include phosphatidic acid, phosphatidylserine, phosphatidylinositol and phosphatidylglycerol, among other phospholipids. Of those phospholipids that are not negatively charged, phosphatidylcholine and phosphatidylethanolamine predominate. Preferably, the weight ratio of phosphatidylcholine to phosphatidylethanolamine in the phospholipid mixture is about 1:1, but may vary over a wide range. The amount of phospholipid mixture included in the compositions of the present invention varies, but generally ranges between about 5% and about 40%, preferably 20% to 40%, of the total weight of phospholipids and neutral lipids in the composition. The amount of the phospholipid mixture included in the compositions of the present invention is such that the total amount of negatively charged phospholipids is at least about 10% by

weight of the total weight of phospholipids and neutral lipids.

The inclusion of relatively large amounts of negatively charged phospholipids is viewed as important to the activity of the compositions. By including large amounts of negatively charged phospholipids above about 10% of the total weight of phospholipids and neutral lipids, compositions of the present invention may act to provide a source of negatively charged phospholipids which disappear during the aging process, especially in the brain. The inclusion of high percentages of negatively charged phospholipids in supplement compositions of the present invention acts to reverse this disappearance.

Compositions of the present invention also comprise a neutral lipid mixture obtained from a non-egg yolk source. The amount of neutral lipid included in compositions of the present invention ranges from about 20% to about 80% of the total weight of phospholipids and neutral lipids in the composition. Preferably, compositions of the present invention comprise about 30 to about 50% neutral lipids. Neutral lipids include mono-glycerides, di-glycerides and tri-glycerides as well as cholesterol, carotene and other hydrophobic pigments. Preferable sources of neutral lipids include butter, fish oil and cocoanut oil. While a number of mixtures of neutral lipids may be used in practicing the present invention, it is preferred that a source of neutral lipids which comprises predominantly (greater than 50% by weight) short-chain neutral lipids is used. Short-chain neutral lipids are mono, di, and tri-glycerides having side chains of less than 12 carbon units and include, for example glycerol tributyrate (tributyrene). Short-chain neutral lipids are preferred because they function as potent precursors for phospholipid and neutral lipid synthesis.

It is preferred that the neutral lipid mixture comprises about 30% to about 50% by weight of the total weight of phospholipids and neutral lipids. The amount of neutral lipid incorporated into compositions of the present invention is chosen so that the weight ratio of neutral lipid to phospholipid ranges between about 1:1 to about 2.5:1. It is especially preferred that the weight ratio of neutral lipids

to phospholipids in compositions of the present invention is about 2:1.

Compositions of the present invention may include a physiologically compatible antioxidant, for example, alpha-tocopherol, tocopherylacetate and ascorbic acid, among others. It is preferred that the antioxidant comprises between about 0.2% to about 2.0% by weight of the phospholipids and neutral lipids, and, most preferably, the antioxidant comprises about 0.5% by weight of the phospholipids and neutral lipids.

Ethyl alcohol is preferably included in liquid dietary supplements formulated with compositions of the present invention. In the liquid supplement, the alcohol preferably comprises at least about 0.5% v/w of the liquid supplement. The alcohol is included in the supplement to aid the emulsification of the lipid mixtures (phospholipids and neutral lipids), to provide preservative and anti-bacterial activity and to function as a fluidizer in conjunction with the lipid mixtures. The ethyl alcohol appears to act synergistically with lipid mixture compositions of the present invention.

Compositions of the present invention are preferably provided as a liquid dietary supplement or alternatively, these compositions may be formulated as granules, capsules or suppositories. The liquid supplement may include a number of suitable carriers and additives including water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents and the like. In capsule, granule or suppository form, the compositions of the present invention are formulated in admixture with a pharmaceutically acceptable carrier.

To prepare the compositions of the present invention in capsule, granule or suppository form, one or more compositions of the present invention may be intimately admixed with a pharmaceutically acceptable carrier according to conventional formulation techniques. For solid oral preparations such as capsules and granules, suitable carriers and additives such as starches, sugars, diluents, granulating agents, lubricants, binders, disintegrating agents and the like may be included.

Compositions of the present invention are ingested in liquid form or are taken in capsule, granule or suppository form once or twice a day. Dosages generally should range from

about 10 to 50 g. per day depending on the composition ingested. In the most preferred embodiment of the present invention, the daily dose should be about 24 g. per day. The compositions are preferably ingested once daily in the morning or the evening on an empty stomach or with non-fat foods at least two hours before eating.

The present invention also relates to methods for alleviating the occurrence and severity of hot flushes associated with estrogen withdrawal in menopausal women and for slowing the deterioration of mental processes and improving memory, especially short-term memory.

In the methods of the present invention, the lipid mixture compositions of the present invention may be used to alleviate the occurrence and severity of hot flushes in menopausal women. These same compositions may be used to improve short-term memory and other mental dysfunction caused by the process of aging. In a further embodiment of the present invention, the commercially available egg lecithin product Al-721™ is used to alleviate hot flushes in women and to restore the short-term memory to individuals with mental dysfunction produced by the aging process.

In the method of the present invention compositions of the present invention, as well as AL-721™, are provided as a dietary supplement in doses of about 24 grams of total lipid for periods of about two weeks to individuals exhibiting hot flushes or loss of short-term memory. The method of the present invention results in alleviation of the debilitation associated with these conditions.

AL-721™ egg lecithin is an lipid mixture extract from egg yolks comprising about 70% of a neutral lipid fraction, and about 30% of phospholipids (phosphatidylcholine and phosphatidylethanolamine in a weight ratio of about 2:1).

The present invention may be further understood by way of the following examples. It is to be understood by those skilled in the art that the following examples are illustrative of practicing the present invention, but in no way should they be considered limiting.

Example 1

A liquid dietary preparation was prepared as follows: Twenty egg-yolks (360 g.) were introduced into 6 liters of ethyl alcohol (95%) and boiled for one hour. The heated mixture was filtered to remove undesired residues. To the resulting liquid was added 100 grams of soya lecithin (commercial granulated soya lecithin, about 70% phospholipids), 100 grams of butter and 2 grams of vitamin E as an anti-oxidant. The mixture was thoroughly homogenized and the alcohol content was evaporated to a residual alcohol content of from about 10 to about 20%. The recovered alcohol was recycled and used with additional batches. To the residual material was added a quantity of water up to a final volume of 3 liters. Additives including flavoring such as vanilla or cocoa, coloring and additional additives may be used to increase the palatability of the formulation. Formulations having a low alcohol content have a pleasant taste.

The final product is an aqueous emulsion which includes about 12% (w/v) of lipids as the active ingredient, 5-8% ethyl alcohol and a mixture of antioxidants.

Example 2

The weight ratio of the egg-yolk lipid mixture, the phospholipid mixture and the neutral lipid mixture may be varied according to the scope of the present invention to produce products containing the following weight ratios of the lipid components:

Component	Amount Percent by Weight of Total Lipid Mixture
a) lipid mixture from egg yolk	5 to 40%
b) phospholipid mixture from vegetable source containing at least about 30% by weight of negatively-charged phospholipids	5 to 40%
c) neutral lipids	20 to 80%
d) antioxidant	0.2 to 2.0%

These compositions may be formulated as a liquid dietary supplement containing preferably at least about 0.5% v/w of the liquid supplement and, most preferably, about 5% to about 8%, along with flavorings, colorings and the like, or alternatively, these compositions may be formulated using standard pharmaceutical formulation techniques to produce a dietary supplement in granule, capsule or suppository form.

Example 3

Effect of Lipid Mixture Compositions On Hot Flushes of Menopausal Women

The composition of Example 1 was tested in a group of 12 women who were measured for the number and intensity of daily hot flushes. Each of the women was subjected to 4 test periods with an interval between each of these periods. For period A, the number and intensity of hot flushes were measured in the group of 12 women who had not received the composition of Example 1. For period B, the number and intensity of hot flushes were measured in the same group of 12 women after ingesting a cocktail mixture comprising 12 g. of lipid mixture (100 ml. of Example 1) daily for a period of 2 weeks. For period C, the number and intensity of hot flushes in the group of 12 women were measured after ingesting a cocktail mixture comprising 24 g. of lipid mixture. For period D, the number and intensity of hot flushes were measured 3 to 10 days after the lipid mixture compositions were withdrawn.

Results of the test (Table 1) demonstrate a pronounced decrease in the severity of the flushes, as well as in their total number. After termination of the administration of the liquid dietary supplement of Example 1 (period D), a partial relapse occurred.

Table 1: Daily Number and Intensity of Hot Flushes in 12 Women

Intensity/ Period	Mild	Moderate	Severe	Total	Weighted Mean ±S.D.
A	0.75	5.0	10.5	6.3	42.3 ±15.5
B	3.5	2.5	2.5	8.5	16.0 ± 9.0
C	3.5	1.5	2.3	7.3	13.4 ±10.0
D	2.5	4.8	4.2	11.5	24.7 ±11.0

Period of Test:

A- control
B- 12 g. Cocktail Mixture (Example 1) daily for 2 weeks
C- 24 g. Cocktail mixture (Example 1) daily for 2 weeks
D- 3 to 10 days after withdrawal of medication

Each of the women passed 4 sequential periods of tests as indicated above. Hot flushes were scored by the participating subjects using three parameters: temperature, sweat and tremors. The results show a marked reduction in the severity of the flushes, as well as in their total number. Shortly after withdrawal of medication, a partial relapse was observed.

The serum lipid components including cholesterol, triglycerides, high density lipoproteins (HDL) and low density lipoprotein (LDL) of the 12 women were determined during the trial periods. These are presented in Table 2, below. The results demonstrate that no substantial change of serum lipids took place.

The levels of the hormones FSA and beta-estradiol in the plasma were also determined. The results, summarized in Table 3, indicate that there was no appreciable change in hormone levels as a result of the ingestion of these lipids.

Table 2: Serum Components (mg/ml) (Mean + S.D.) During The Study Period (1st Trial)

Serum Components	PERIOD A	B	C	D
Total Cholesterol	252	256	258	268.5
Triglycerides	165	178	227	207.7
Total Cholesterol	4.8	4.9	5.0	4.5
HDL	60	57	55	62
LDL	3.0	2.9 ± 0.3	3.0 ± 0.5	2.7

Period of Test:

A- control
B- 12 g. Cocktail Mixture (Example 1) daily for 2 weeks
C- 24 g. Cocktail mixture (Example 1) daily for 2 weeks
D- 3 to 10 days after withdrawal of medication

Table 3: Mean Values of FSH (mU/ml) and 17 beta-estradiol (pg/ml) During Study Period (First Trial)

Period	FSH	17 beta-estradiol
A	99	33
B	106	32
C	98	35
D	100	31

Period of Test:

A- control
B- 12 g. Cocktail Mixture (Example I) daily for 2 weeks
C- 24 g. Cocktail mixture (Example 1) daily for 2 weeks
D- 3 to 10 days after withdrawal of medication

Example 4

Effect of Lipid Mixture Composition AL-721™ On Hot Flushes of Menopausal Women

The lipid mixture composition AL-721™ was tested in a group of 10 women who were measured for the number and intensity of daily hot flushes. Each of the women was subjected to 3 test periods with an interval between each of these periods. For period A, the number and intensity of hot flushes were measured in women who had not received AL-721™. For period B, the number and intensity of hot flushes were measured in the same group of 10 women after ingesting 15 g. of AL-721™ daily for a period of 2 weeks. For period C, the number and intensity of hot flushes were measured 3 days after AL-721™ was withdrawn.

Results of the test demonstrate (Table 4) a significant decrease in the severity of the flushes, as well as in their total number. After termination of the administration of AL-721™ (period C), a partial relapse of hot flushes occurred.

Table 4: Average Daily Number and Intensity of Hot Flushes in 10 Women Receiving AL-721™

	INTENSITY			
Period	Mild	Moderate	Severe	Total
A	1.2	4.6	8.6	14.4
B	2.2	3.6	1.6	7.4
C	3.5	3.2	3.8	10.5

Periods of Test:

A- before treatment
B- 15 g. of AL-721™ daily for 2 weeks
C- 3 days after withdrawal of AL-721™

This trial was carried out several months after trial number 1 (Example 3). Each of the women passed through three sequential periods of test as indicated. The results are similar to those presented in Table 1 and verify the use of AL-721™ for use as an agent to reduce hot flushes in menopausal women.

Example 5

Effect of Example 1 Composition on Short Term Memory

Studies have shown that after ingestion of 24 g. of the composition of Example 1 for a period of a few weeks, improvement of memory took place and the general well-being of the persons treated improved. Small-scale trials on individuals suffering from loss of short-term memory and other mental dysfunction indicate the pronounced efficacy of the preparations of the invention in this respect. The trials were carried out on an individual basis and results were based on the individuals reporting subjective improvements during the studies.

It is to be understood by those skilled in the art that the foregoing description and examples are illustrative of practicing the present invention, but are in no way limiting. Variations of the detail presented herein may be made without departing from the spirit and scope of the present invention.

CLAIMS:

1. A dietary supplement for ingestion by humans comprising:

a) a lipid mixture obtained from egg yolks by extracting said egg yolks with an organic solvent, said lipid mixture containing a mixture of phospholipids comprising about 5% to about 40% by weight of the total weight of phospholipids and neutral lipids in said supplement;

b) a phospholipid mixture obtained from a source other than egg yolks in an amount equal to about 5% to about 40% by weight of the total weight of phospholipids and neutral lipids in said supplement wherein at least about 30% by weight of the total phospholipid content of said phospholipid mixture is comprised of negatively charged phospholipids; and

c) a neutral lipid mixture obtained from a source other than egg yolks in an amount equal to about 20% to about 80% by weight of the total weight of phospholipids and neutral lipids in said supplement, and wherein said lipid mixture, said phospholipid mixture, and said neutral lipid mixture together comprise at least about 5% by weight of said supplement, said negatively charged phospholipids comprise at least about 10% by weight of said phospholipids and neutral lipids of said supplement and the weight ratio of neutral lipids to phospholipids in said supplement ranges from about 1:1 to about 2.5:1.

2. The dietary supplement according to claim 1 further comprising at least about 0.5% (v/w) of the total weight of the supplement of ethyl alcohol.

3. The supplement according to claim 1 or 2 further comprising an effective amount of a physiologically acceptable antioxidant.

4. The supplement according to any of claims 1 through 3 wherein said antioxidant comprises about 0.2 to about 2.0% by weight.

5. The supplement according to any of claims 1 through 4 wherein said lipid mixture is obtained by the extraction of egg yolks with boiling 95% ethyl alcohol.

6. The supplement according to any of claims 1 through 5 wherein said phospholipid mixture is soya lecithin.

7. The supplement according to any of claims 1 through 6 wherein said neutral lipid mixture is obtained from animal

butter.

8. The supplement according to any of claims 1 through 7 wherein said neutral lipid mixure is predominantly comprised of short-chain neutral lipids.

9. A dietary supplement for ingestion by humans comprising:

a) a lipid mixture obtained from egg yolks by extracting said egg yolks with boiling ethanol for a period of about one hour, said lipid mixture containing a mixture of phospholipids comprising about 5% to about 40% by weight of the total weight of phospholipids and neutral lipids in said supplement;

b) a phospholipid mixture obtained from a source other than egg yolk in an amount equal to about 5% to about 40% by weight of the total weight of phospholipids and neutral lipids in said supplement wherein at least about 30% by weight of the total phospholipid content of said phospholipid mixture is comprised of negatively charged phospholipids; and

c) a neutral lipid mixture obtained from a source other than egg yolk in an amount equal to about 20% to about 80% by weight of the total weight of phospholipids and neutral lipids in said supplement, and wherein said lipid mixture, said phospholipid mixture, and said neutral lipid mixture together comprise at least about 5% by weight of said supplement, said negatively charged phospholipids comprise at least about 10% by weight of said phospholipids and said neutral lipids.

10. The supplement according to claim 9 wherein the weight ratio of neutral lipids to phospholipids in said supplement ranges from about 1:1 to about 2.5:1.

11. The supplement according to claims 9 or 10 wherein said phospholipid mixture is obtained from soya lecithin and said neutral lipid mixture is obtained from animal butter.

12. A dietary supplement for use in treating hot flushes in menopausal women comprising comprising:

a) a lipid mixture obtained from egg yolks by extracting said egg yolks with an organic solvent, said lipid mixture containing a mixture of phospholipids comprising about 5% to about 40% by weight of the total weight of phospholipids and neutral lipids in said supplement;

b) a phospholipid mixture obtained from a source other than egg yolks in an amount equal to about 5% to about 40% by weight of the total weight of phospholipids and neutral lipids

in said supplement wherein at least about 30% by weight of the total phospholipid content of said phospholipid mixture is comprised of negatively charged phospholipids; and

c) a neutral lipid mixture obtained from a source other than egg yolks in an amount equal to about 20% to about 80% by weight of the total weight of phospholipids and neutral lipids in said supplement, and wherein said lipid mixture, said phospholipid mixture, and said neutral lipid mixture together comprise at least about 5% by weight of said supplement, said negatively charged phospholipids comprise at least about 10% by weight of said phospholipids and neutral lipids of said supplement and the weight ratio of neutral lipids to phospholipids in said supplement ranges from about 1:1 to about 2.5:1.

13. The supplement according to claim 12 wherein said lipid mixture comprises about 20% to about 40% by weight, said phospholipid mixture comprises about 20% to about 40%, and said neutral lipid mixture comprises about 30% to about 50% by weight of said phospholipids and neutral lipids.

14. The supplement according to claims 12 or 13 wherein said supplement further comprises a pharmaceutically compatible antioxidant.

15. The supplement according to any of claims 12 through 14 wherein said supplement further comprises at least about 0.5% v/w of the total weight of the supplement of ethyl alcohol.

16. The supplement according to any of claims 12 through 15 wherein said lipid mixture is obtained by extracting egg yolks with boiling ethanol for a period of one hour.

17. The supplement according to any of claims 12 through 16 wherein said phospholipid mixture is soya lecithin.

18. The supplement acording to any of claims 12 through 17 wherein said neutral lipid mixture is obtained from animal butter.

19. The supplement according to any of claims 12 through 18 wherein said neutral lipid mixture is predominantly comprised of short-chain neutral lipids.

20. A dietary supplement for use in treating mental dysfunction including loss of short-term memory caused by the aging process in humans comprising:

a) a lipid mixture obtained from egg yolks by extracting

said egg yolks with an organic solvent, said lipid mixture containing a mixture of phospholipids comprising about 5% to about 40% by weight of the total weight of phospholipids and neutral lipids in said supplement;

b) a phospholipid mixture obtained from a source other than egg yolks in an amount equal to about 5% to about 40% by weight of the total weight of phospholipids and neutral lipids in said supplement wherein at least about 30% by weight of the total phospholipid content of said phospholipid mixture is comprised of negatively charged phospholipids; and

c) a neutral lipid mixture obtained from a source other than egg yolks in an amount equal to about 20% to about 80% by weight of the total weight of phospholipids and neutral lipids in said supplement, and wherein said lipid mixture, said phospholipid mixture, and said neutral lipid mixture together comprise at least about 5% by weight of said supplement, said negatively charged phospholipids comprise at least about 10% by weight of said phospholipids and neutral lipids of said supplement and the weight ratio of neutral lipids to phospholipids in said supplement ranges from about 1:1 to about 2.5:1.

21. The supplement according to claim 20 wherein said lipid mixture comprises about 20% to about 40% by weight, said phospholipid mixture comprises about 20% to about 40%, and said neutral lipid mixture comprises about 30% to about 50% by weight of said phospholipids and neutral lipids.

22. The supplement according to claims 20 or 21 wherein said supplement further comprises a pharmaceutically compatible antioxidant.

23. The supplement according to any of claims 20 through 22 wherein said supplement further comprises at least about 0.5% v/w of the total weight of the supplement of ethyl alcohol.

24. The supplement according to any of claims 20 through 23 wherein said lipid mixture is obtained by extracting egg yolks with boiling ethanol for a period of one hour.

25. The supplement according to any of claims 20 through 24 wherein said supplement is administered to said human for a period of at least two weeks.

26. A method of treating hot flushes in menopausal women comprising administering to a woman experiencing hot flushes at

least about 15 g. daily of AL-721™ for a period of at least about two weeks.

27. A method of treating mental dysfunction including loss of short-term memory caused by the aging process in humans comprising administering to an individual experiencing a loss of memory at least about 15 g. daily of AL-721™ for a period of at least about two weeks.

28. A method of making a dietary supplement for ingestion by humans comprising:

a) extracting egg yolks with an organic solvent to produce a lipid mixture containing a mixture of phospholipids and neutral lipids comprising about 5% to about 40% by weight of the total weight of phospholipids and neutral lipids in said supplement;

b) adding to said egg yolk

i) a phospholipid mixture obtained from a source other than egg yolks in an amount equal to about 5% to about 40% by weight of the total weight of phospholipids and neutral lipids in said supplement wherein at least about 30% by weight of the total phospholipid content of said phospholipid mixture is comprised of negatively charged phospholipids and

ii) a neutral lipid mixture obtained from a source other than egg yolks in an amount equal to about 20% to about 80% by weight of the total weight of phospholipids and neutral lipids in said supplement, and wherein said lipid mixture, said phospholipid mixture, and said neutral lipid mixture together comprise at least about 5% by weight of said supplement, said negatively charged phospholipids comprise at least about 10% by weight of said phospholipids and neutral lipids of said supplement and the weight ratio of neutral lipids to phospholipids in said supplement ranges from about 1:1 to about 2.5:1; and

c) homogenizing said lipid mixture, said phospholipid mixture and said neutral lipid mixture.

29. The method according to claim 28 comprising the additional step of adding a pharmaceutically compatible antioxidant to said mixture from claim 28.

30. The method according to claims 28 or 29 comprising the additional step of adding ethyl alcohol to said mixture from claim 28.

31. A method of making a dietary supplement for ingestion by humans comprising:

a) extracting egg yolks with boiling ethyl alcohol for a period of about one hour to produce a lipid mixture containing a mixture of phospholipids and neutral lipids comprising about 5% to about 40% by weight of the total weight of phospholipids and neutral lipids in said supplement;

b) filtering said mixture to remove undesired residues;

c) adding to said egg yolk mixture

i) a phospholipid mixture obtained from a source other than egg yolks in an amount equal to about 5% to about 40% by weight of the total weight of phospholipids and neutral lipids in said supplement wherein at least about 30% by weight of the total phospholipid content of said phospholipid mixture is comprised of negatively charged phospholipids and

ii) a neutral lipid mixture obtained from a source other than egg yolks in an amount equal to about 20% to about 80% by weight of the total weight of phospholipids and neutral lipids in said supplement, and wherein said lipid mixture, said phospholipid mixture, and said neutral lipid mixture together comprise at least about 5% by weight of said supplement, said negatively charged phospholipids comprise at least about 10% by weight of said phospholipids and neutral lipids of said supplement and the weight ratio of neutral lipids to phospholipids in said supplement ranges from about 1:1 to about 2.5:1;

c) homogenizing said lipid mixture, said phospholipid mixture and said neutral lipid mixture; and

d) evaporating said ethyl alcohol to about 10% to about 20% of said ethyl alcohol used for extracting said egg yolks.

32. The method according to claim 31 comprising the additional step of adding a pharmaceutically compatible antioxidant to said mixture from claim 31.

INTERNATIONAL SEARCH REPORT

International Application No PCT/NL 88/00058

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: A 61 K 37/22; A 23 L 1/30; A 23 J 7/03; C 11 B 9/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System [8]	Classification Symbols
IPC[4]	A 61 K; A 23 L; A 23 J; C 11 B

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [9]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	EP, A, 0074251 (YEDA RESEARCH AND DEVELOPMENT CO., LTD) 16 March 1983 see claims 1-12,14,16; page 9, lines 20-34 & US, A, 4474773 (cited in the application)	1,3,4,6, 9,12,17, 20-22,28, 31,32
A	EP, A, 0148303 (VON MLETZKO, ARMIN) 17 July 1985 see claims 1-9; page 5, lines 13-30; page 2, lines 13-26	1,6,8,9, 12,13,17, 19,20,21, 28,31,32
A	EP, A, 0185235 (A. NATTERMAN & CIE. GmbH) 25 June 1986 see claims 1-5	1,9,12,20, 28
A	EP, A, 0141442 (UNILEVER PLC) 15 May 1985 see claims 1-20	1,9,12,20, 28

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
12th April 1989	09 MAY 1989
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers * because they relate to subject matter not required to be searched by this Authority, namely:

* 25,26,27

See PCT Rule 39.1(iv): methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods

2. [ ] Claim numbers ..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers ..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. NL 8800058 SA 25717

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 28/04/89 The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0074251	16-03-83	AU-A- 8778282	10-03-83
		JP-A- 58126812	28-07-83
		US-A- 4474773	02-10-84
		US-A- 4677099	30-06-87
EP-A- 0148303	17-07-85	None	
EP-A- 0185235	25-06-86	DE-A- 3445950	19-06-86
		JP-A- 61145189	02-07-86
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		DE-A- 3469672	14-04-88